Impact Investigation of a Mercury Reducing GEM in Stream Microcosms
&
Construction of Mercury Reducing Reporter Strains Based on the Safety Strain *Ps. putida* KT2440

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Wagner-Döbler, I., von Canstein, H. F., Leonhäuser, J., Li, Y., Felske, A., Pauling, B. V.: Mercury remediation- a new solution to an old problem. 6^{th} International Conference on Mercury as a Global Pollutant, October 15-19, Minamata, Japan (2001).

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The insults to humanity through science

1. *The astronomical insult*
    - the planet earth is not the centre of the universe

2. *The genetic insult*
    - humankind is not the centre of the living world
    - the genetic code is universal
    - the enzymes and biochemical pathways are universal
    - sequence homologies exist
    - horizontal gene transfers may take place

3. *The psychological insult*
    - our mind is split into the conscious and the unconscious
    - in case of disagreement, the unconscious always wins

(E. Kellenberger, 1994)

Contents

1 Introduction

1.1 Bacterial Bioremediation of Mercury Contaminated Water

1.1.1 Mercury in the Environment

Mercury has been present in the environment for aeons. Erupted from the centre of the earth by volcanic activity mercury exists as mineral (mainly as cinnabar -red HgS-, but also as mercury oxide, oxychloride, sulfate mineral; Kim *et al.* 2001) or as elemental mercury, the only metal to be a liquid at room temperature but also existing as gas due to its high vapour pressure. In a bio-geo-chemical cycle mercury is globally dispersed undergoing many physical and chemical transformations (Barkay 2000): (1) In the atmosphere elemental mercury is photo-oxidised to ionic mercury (Hg^{2+}). (2) Rain precipitates the anorganic mercury on the surface of the earth where, carried out mainly by microorganisms in aquatic systems, it is (3) reduced back to its elemental form or (4) methylated. (5) Elemental mercury evaporates into air where the cycle begins anew. Mercury is emitted by four main natural processes: (1) degassing from geological mineral deposits, (2) emissions from volcanic activities, (3) photoreduction of divalent mercury in aquatic systems, (4) biological formation of elemental and methyl-mercury (OECD/GD(94)98).

Although it remains undisputed that mercury occurs naturally in the environment and in toxic concentrations locally, mercury emissions owing to anthropogenic activities in industry (mainly through chlor-alkali-electrolysis), mining (Korte 1999), and fossil fuel combustion or waste incineration are immense and are thought to substantially contribute to the mercury pool participating in the bio-geo-chemical cycle. While natural mercury emission is estimated at 1000 t y^{-1}, 2000 t of industrial mercury are assumed to be emitted per year (Mason *et al.* 1994). However, the concentrations of mercury in various compartments from natural and anthropogenic sources are highly variable. Thus, estimations for mercury emissions to air have previously ranged from 2500 to 125,000 t y^{-1} (OECD/GD(94)98).

Worldwide many areas are mercury polluted and present a threat to people and environment (Fukuda *et al.*, 1999; Cleckner *et al.* 1999, Xiao *et al.* 1998, Horvat *et al.* 1999). Owing to the mercury cycle described above, regional emissions may be deposited elsewhere, e.g. in the Arctic where increased mercury levels have been found (Macdonald *et al*. 2000), hence contaminations do not remain confined to a particular area but may affect ecosystems globally.

Via the food chain, and most efficiently in the aquatic food web, mercury (mostly as methylmercury) accumulates in carnivorous fish, a process called biomagnification, poisoning people consuming the fish. The syndromes (neurological disorders, see below) resulting from the poisoning have been named Minamata Disease after the tragedy in the late 1950s when over 3000 people in Japan were severely poisoned by methylmercury pollution caused by a chemical manufacturing plant (Langford & Ferner 1999).

1.1.2 Toxicity of Mercury

Mercury is known to most people in the western world from every day life. Before the prohibition by the European Union directive (European Council Directive 93/42/EEC) it was widely used in clinical thermometers, moreover mercury can be found in several medications and it is still used in dental fillings nowadays. However, its toxicity has also been known and recorded for two milleniums (Langford & Ferner 1999).

Different forms of the heavy metal possess different characteristics rendering it a hazard to living organisms. The toxicity of highly reactive mercuric mercury (Hg^{2+}) is attributed to it binding to the sulfhydryl groups of the cysteines of essential enzymes and proteins, thus disturbing vital cell functions. In the body water-soluble ionic mercury salts are not efficiently absorbed. Rather, they are eliminated from the body via the kidneys acutely causing damage to the gut and the renal system in cases of poisoning. Hg^{2+} is generated in large amounts in mercury cells during chlor-alkali electrolysis. Waste water from this industrial process that produces chlorine and caustic soda normally contains between 1 and 10 ppm of mercury.

The hazard arising from elemental mercury (Hg^0) is owed to its high vapour pressure allowing it to be easily inhaled. Absorbed by the lungs it enters the blood stream and is distributed around the body including the brain. Elemental mercury is transformed in the red blood cells, the liver and the central nervous system to Hg^{2+} and methylmercury. Repeated or prolonged exposure mainly results in vasomotor disturbances, tremor, and behavioural disturbances. Organic mercury forms such as monomethyl- or dimethylmercury or phenyl mercuric acetate (PMA) are lipid-soluble and thus readily absorbed in the body. They penetrate membranes and cross the blood brain barrier. A large proportion of organic mercury is transformed into reactive Hg^{2+} (Strasdeit 1998) and can severely damage the central nervous system causing neuromuscular malfunctions, ranging from numb limbs and visual disorders to paralysis and even death (Barkay 2000). Because transformation to Hg^{2+} occurs slowly, symptoms of poisoning with organic mercury may appear weeks or months after the poisoning as in the case of Karen Wetterhahn, a chemistry professor who tragically died after she had spilled not

more than a drop or two of dimethylmercury on her hand that she supposed to have protected with a latex glove. She did follow safety procedures immediately after the incidence. First symptoms of neurological impairment did occur no earlier than 5 months after the accident, she passed away 10 months after the spill (Nierenberg *et al*. 1998).

1.1.3 Bacterial Resistance to Mercury

Mercuric mercury forms complexes with organic and inorganic ligands and easily adsorbs to surfaces of particulates owing to its high reactivity and affinity to thiol groups (Barkay 2000). Mercury bio-availability therefore plays a crucial role in the evaluation of microbial resistance levels. There is some tolerance towards mercury owing solely to unspecific sequestration by cells walls and lipopolysaccharide (LPS) layers (Langley & Beveridge 1999).

Bioaccumulation of mercury by means of active transport of the mercury ions into the cell and subsequent tethering by metallothioneins has not been observed in natural bacteria (Osborn *et al*. 1997). Metallothioneins are small proteins with a strong metal-binding capacity by virtue of their cysteine-residues. They are ubiquitous in the plant and animal kingdom and have therefore been of interest in the construction of bacterial strains for bioremediation of mercury (Hg^{2+}) contaminated waste water (Chen *et al*. 1998, Valls *et al*. 2000).

True mercury resistance is characterized by active enzymatic detoxification. Among bacteria mercury resistance is ancient, highly conserved and has been identified across a wide range of bacterial genera including gram positive and gram negative bacteria. Bacteria resistant to mercury have been isolated world-wide from a diverse range of habitats and locations (Osborn *et al.* 1997). The genes encoding it are organised within the so-called *mer* operon and can be found even in microorganisms that have not particularly been isolated from mercury-contaminated sites, although the frequency of *mer* genes at these sites is lower than in the presence of mercury (Barkay *et al*. 1991, Rasmussen & Sørensen 1998, Smit *et al*. 1998). Some *mer* operons have been found to be encoded chromosomally (Inoue *et al*. 1989, Wang *et al*. 1989, Iohara *et al*. 2001), however most have been isolated from (self-transmissible) plasmids (Osborn *et al*. 1997, Rasmussen & Sørensen 1998, Smit *et al*. 1998). The operon can also be spread by transposition and has been found on several natural transposons (Liebert *et al*. 1997, Liebert *et al.* 1999, Hobman *et al*. 1994, Misra *et al.* 1984). Horizontal gene transfer by transposition is presumed to be the reason for the presence of two or more *mer* operons in one strain of *Bacillus* (Gupta *et al.* 1999, Huang *et al*. 1999). The mercury resistance operon comprises three major functions: Transport of Hg^{2+} into the cell, enzymatic NADPH-dependent conversion of the ionic mercury into relatively non-toxic elemental mercury (Hg^0)

and the regulation of the genes (Misra 1992, Cervantes & Silver 1996). Resistance operons restricted to these functions are termed "narrow spectrum". The detoxification of organic mercury requires a fourth function, namely cleavage of mercury from the organic residue. If this is included in the mercury resistance operon it is labelled "broad spectrum". The genes conferring these functions are designated *mer*T, *mer*P (transport), *mer*A (mercury reduction), *mer*B (cleavage of Hg from organic residue), *mer*R, and *mer*D (regulatory genes). Further *mer* genes have been identified recently: *mer*C and *mer*F, both membrane proteins, confer transport functions (Wilson *et al*. 2000), while the *mer*G product provides resistance to phenylmercury (Kiyono & Pan-Hou 1999). Most mercury resistance operons are inducible, i.e. Hg^{2+} has to be present in order to activate expression of the resistance whereas transcription is suppressed in the absence of mercury (Misra 1992). If, however, switched on by mercury at a contaminated site, the bacterial mercury resistance forms the basis of natural on-site detoxification of mercury (Silver *et al*. 1994) under aerobic conditions.

1.1.4 Utilizing the Bacterial Mercury Resistance in Bioremediation

The ability of bacteria to detoxify mercury can be utilized to bioremediate mercury contaminated waste water. In fact, it seems as if this potential was solely confined to microbial species as no naturally occurring plant or animal species have been reported to detoxify mercury. Nevertheless, plants have been engineered to overexpress the bacterial mercury resistance and transform organic and inorganic mercury to elemental mercury with promising results for the phytoremediation of mercury contaminated sites (Rugh *et al.* 1998, Bizily *et al.* 1999). Plants combine many practical benefits for the bioremediation of contaminated sites. With the mercury transforming transgenic yellow poplar of Rugh *et al*. 1998, large areas of contamination could be planted. In addition to decontamination, the trees would stabilize the soil surface with a stout root systems and provide niches for the inhabitation of (mercury-reducing) microorganisms. However, the mercury reducing plants emit volatile Hg^0 into the air, which although non-significant on a global scale may contribute to increased mercury concentrations locally.

One of the initial efforts to retain mercury in bacterial bioreactors was made by Brunke *et al*. (1993). They managed to capture elemental mercury in globules up to 5 µm in diameter in fixed-bed columns using genetically engineered mercury reducing bacteria that were immobilized on ceramic carriers, glass or in alginate beads. Some years later, von Canstein *et al.* (1999) demonstrated the removal of mercury from chloralkali electrolysis waste water by a mercury resistant *Pseudomonas putida* strain. This natural isolate was capable of coping with

up to 8 mg L^{-1} of mercury in the waste water, transforming 97.3% (cellulose fibres) or 98.5% (Siran), depending on the carrier material. These laboratory-scale reactor results formed the basis for the development of a technical-scale bioreactor that de-contaminated mercury-polluted chlor-alkali-electrolysis waste water under on-site conditions (Wagner-Döbler *et al*. 2000a&b). Mercury retention could be achieved at 95% efficiency, and the discharge limit for mercury in industrial waste water (50 µg L^{-1}) could be met reliably with the help of an activated carbon filter at the end of the line, capturing residual traces of mercury. The elemental mercury accumulated in the bioreactor, this, however, did not affect the efficacy of the bioreactor.

Bioremediation exploiting the bacterial resistance system is very cost-effective in comparison with chemical methods of mercury-decontamination such as sulfide precipitation or ion exchange columns. All it requires in addition to the bacteria is a sucrose feeding solution from which they can derive the energy needed for the reduction of mercury. In the flow-through reactor dead cells are flushed from the system and are constantly replaced by fresh re-growth.

It was found that although inoculated with several mercury reducing isolates, foreign mercury-resistant bacteria invaded the bioreactor and a new consortium of mercury-transforming bacteria evolved that dynamically changed over time (von Canstein *et al*. 2002b) having to cope with temporary high inflow mercury concentrations, temperature increase, oxygen and nutrient gradients and fluctuating concentrations of chloride. The efficiency of mercury reduction, however, was at no stage impaired by the changing bioreactor community. Some of the original inoculants could be detected throughout the time of operation, others became temporarily non-detectable and re-appeared at later stages. In additional experiments in the laboratory-scale bioreactors, the presence of a consortium could be shown to be of benefit for a reliable, disturbance-independent mercury-removal (von Canstein *et al*. 2002a). Due to the continuous selective pressure in the bioreactor, mercury resistant bacteria are enriched that are most properly adapted to on site conditions and thus best suited to improve bioreactor stability.

1.1.5 Genetically Engineered Microorganisms (GEMs) in Biotechnology and Bioremediation

Recombinant microorganisms have proven very valuable for biotechnology for decades. By now they are indispensable for the production of amino acids, vitamins, alcohol, antibiotics or secondary metabolites for health and nutrition (Demain 2000). Furthermore, bacteria have been isolated and engineered to biodegrade or detoxify pollutants (Barkay & Schaefer 2001).

Industrialization has taken its toll by leaving many environmental habitats, especially rivers, polluted and ecosystems severely damaged. However, bacteria have had an aeons lasting history of adaptation to adverse conditions, that have forced upon them the capacity to cope with pollutants, having already evolved a detoxifying mechanism or developing one under the selective pressure of the toxin, e.g. by expanding the substrate range of an enzyme that is already present (de Lorenzo 2001). Recombinant DNA technology has provided a tool for accelerating this process in a rational way. The improvement of microbes that appear promising for bioremediation may be achieved e.g. by creating new metabolic routes, by optimising substrate routing, improving turnover rate or enhancing process-relevant properties of the microorganism (Timmis & Pieper 1999, Pieper & Reineke 2000).

1.1.5.1 GEMs in Mercury Bioremediation

Genetic modification of microorganisms for the possible use of mercury-remediation has aimed at increasing mercury resistance by multiplying *mer* operon copy number in *E. coli* and *Ps. putida* (Kurabayashi *et al*. 1997), providing strains already holding special functions, such as the radiation resistant *Deinococcus radiourans,* with the mercury resistance (Brim *et al*. 2000), combining the transport system of the mercury resistance and metallothionein for biosorption in one recombinant *E. coli* strain (Chen *et al*. 1998), or expressing metallothionein on the cell surface of *Ralstonia metallidurans* or *Ps*. *putida* (Valls *et al*. 2000).

The bacterial strain *Ps. putida* KT2442::mer73 is another example for a mercury reducing genetically engineered microbe. It was constructed by Horn *et al.* (1994) and selected for its high and constitutive mercury resistance. The rationale was that the construct strain would be better adapted to cope with fluctuating mercury concentrations than natural bacteria with an inducible mercury resistance. The mercury resistance used in this manipulation was taken from the natural plasmid pDU1358 from *Serratia marcescens* and integrated into the *Ps. putida* KT2442 genome by mini-Tn5-transposon mutagenesis without transfer of the transposase, thus integrating only the necessary genes stably into the chromosome. Being a candidate for bioremediation, *Ps. putida* KT2442::mer73 was tested in the laboratory scale bioreactors described above in a monospecies mercury-reducing biofilm (von Canstein *et al*. 2002a). In this and previous experiments with non-sterile mercury-containing waste water (Felske *et al.* 2001), the GEM was readily lost and did not seem to establish good biofilms in the bioreactor unless feeding was increased. Insufficient growth and a prolonged lag-phase were probably the reason for this.

1.1.5.2 Rationales for New Mercury Reducing Construct Strains

For *Ps. putida* KT2442::mer73 it was shown that high mercury reduction was not sufficient for good performance in the bioreactors. In contrast, mixed culture biofilms consisting of several mercury-resistant isolates efficiently retained mercury and were not affected by disturbances such as rapid increases of mercury or continuously high mercury concentrations. Von Canstein *et al*. (2002a) impressively showed that a moderately mercury reducing isolate ("Bro62") that did not perform well under mono-species conditions, dominated the very successful mercury reducing multi-species biofilm, where it was probably protected by better resistant cells that, however, were only present in very low abundance. With regard to microorganisms used for bioremediation this shows that besides creating a super-organism by combining several features in one strain, a consortium of GEMs with different abilities (which could also include natural bacteria) could be of value for a successful bioremediation strategy, especially if microorganisms in the bioreactor face changing conditions, that may require different abilities.

Bacteria used in packed bed bioreactors in industrial applications need to establish good biofilms and cope well with disturbances (temporary high inflow mercury concentrations, temperature increase, oxygen and nutrient gradients and fluctuating concentrations of chloride). For the construction of new mercury-reducing GEMs to be used in such bioreactors these are traits to decide on. A new highly mercury resistant GEM could be used in combination with a natural consortium, playing a bio-protective role, e.g. in case of sudden mercury peaks in the bioreactor inflow (for bio-protection of an activated sludge microbial community during pollutant shocks see Eichner *et al.* 1999). A GEM's task could also be to fortify the integrity of the biofilm, facilitating attachment of other mercury transforming community members.

Easy monitoring of mercury reduction within the bioreactor is crucial to evaluate the performance of the strain. On-line determination of mercury can serve as indirect measure for the activity of the mercuric reductase, however only the performance of the bioreactor as a whole can be represented in that way and discrimination of individual strains is not possible. By transcriptional fusion of the mercury resistance with a reporter gene (e.g. GFP, see below), the activity of the mercury reduction of a new GEM can be monitored directly. The same reporter can also be used to monitor the fate of the GEM within the bioreactor or in case of escape outside of it. This work describes the construction of mercury reducing *Ps. putida* strains by random Tn5 mutagenesis with a promoterless *mer-gfp* cassette. Details of the recipient strain and the reporter gene used are described in 1.2 and 1.3.2.2 respectively.

1.2 Safety Aspects in the use of GEMs

1.2.1 European Guidelines

By definition a "[…] genetically modified organism (GMO) means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; […]" (2001/18/EC). Since the first utilization of GMOs for industrial processes or in agriculture, there has been discussion and to a great extent concern and scepticism about the safety of the newly created strains in case of an accidental or deliberate release into the environment (e.g. Stotzky & Babich 1984, Wöhrmann 1991, Kellenberger 1994, Sheridan 2000). In the following the abbreviation GMO will be used to refer to all modified organisms while GEM will only be used in the context of genetically engineered microorganisms. In Europe, these concerns have resulted in legislative consequences in form of a set of regulations and guidelines for the use of GEMs. One of the essential pre-requisites for the use of GEMs are good knowledge and description of the donor and recipient (or parental organism/s) and the vector/s used in the construction. Approval of the deliberate release of a GEM requires documented description of its ecology and environmental impact. An environmental risk assessment (e.r.a., Table 1-1) of a release of GEMs requires knowledge of microbial survival, growth, activity and dispersal within the environment, and of the persistence of recombinant DNA and its transfer to the indigenous microflora. Although a variety of microbes have been optimised for bioremediation, these efforts were mainly restricted to the laboratory and confined systems, and only very few data are available from field applications (Sayler & Ripp 2000). For an evaluation of GEMs used in *in situ* bioremediation, however, it is essential that long-term field studies are performed to yield information about the competence of the GEM and the risks associated with its introduction into natural ecosystems.

Table 1-1 Excerpt of the Directive 2001/18/EC. European Parliament and the Council of 12 March 2001, ANNEX II Principles for the Environmental Risk Assessment, D. Conclusions on the potential environmental impact from the release or the placing on the market of GMOs.

> "[…] 1. Likelihood of the GMO to become persistent and invasive in natural habitats under the conditions of the proposed release(s).

2. Any selective advantage or disadvantage conferred to the GMO and the likelihood of this becoming realised under the conditions of the proposed release(s).

3. Potential for gene transfer to other species under conditions of the proposed release of the GMO and any selective advantage or disadvantage conferred to those species.

4. Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO and target organisms (if applicable).

5. Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO with non-target organisms, including impact on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens.

6. Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GMO and persons working with, coming into contact with or in the vicinity of the GMO releases(s).

7. Possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from consumption of the GMO and any product derived from it if it is intended to be used as animal feed.

8. Possible immediate and/or delayed effects on biogeochemical processes resulting from potential direct and indirect interactions of the GMO and target and non-target organisms in the vicinity of the GMO release(s).

9. Possible immediate and/or delayed, direct and indirect environmental impacts of the specific techniques used for the management of the GMO where these are different from those used for non-GMOs."

In summary, the Directive differentiates between "direct" and "indirect effects" referring to primary effects on human health or the environment which are a result of the GMO itself and which do not occur through a causal chain of events, or to effects on human health or the environment occurring through a causal chain of events. Observations of indirect effects are likely to be delayed. Consequently, the Directive includes the time coordinate and recognizes "immediate effects" referring to effects on human health or the environment which are observed during the period of the release of the GMO. Immediate effects may be direct or indirect. "Delayed effects", on the other hand, refer to effects on human health or the environment which may not be observed during the period of the release of the GMO but become apparent as a direct or indirect effect either at a later stage or after termination of the release. Adverse effects may occur directly or indirectly through mechanisms which may include the spread of the GMO(s) in the environment, the transfer of the recombinant genetic material to other organisms, phenotypic and genetic instability, interactions with other organisms or changes in management.

In this work, the mercury-resistant GEM *Ps. putida* KT2442::mer73 was assessed regarding its safety. In accordance with the European Directive 2001/18/EC, survival of the GEM, gene transfer of the foreign genes and its impact on indigenous bacterial communities were especially scrutinized.

1.2.2 Why use *Ps. putida* KT2440 as Parent in the Construction of GEMs?

Ps. putida KT2440 (Bagdasarian *et al*. 1981) is a cured, spontaneous restriction-deficient derivative of *Ps. putida* mt2 (Murray *et al*. 1972) which was isolated from soil by virtue of its ability to use benzoate as the sole source of carbon and which was shown to readily acquire recombinant DNA, expanding its own set of degradative enzymes (Ramos *et al*. 1994). *Ps. putida* KT2440 has been recognized as non-pathogenic by the National Institute of Health of the USA and has been under investigation in a number of physiological and genetic studies (e.g. Ramos-Diaz & Ramos 1998). Its genome has recently been sequenced (Nelson *et al*. 2002, Institute for Genomic Research-TIGR Rockville, MD, USA) and can be obtained now at http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpp, or at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov:80/cgi-bin/Entrez/framik?db=genome&gi=266). The complete sequence elucidates to a large degree the genotype of *Ps. putida* KT2440 and facilitates description of genetic modifications, an aspect that was central for this work. *Ps. putida* KT2440 is easily genetically manipulated (Mermod *et al*. 1986), expresses foreign genes (e.g. Horn *et al*. 1994) and colonizes plant rhizosphere, making it potentially useful for phyto-rhizoremediation and for the development of bio-pesticides (Molina *et al*. 2000). Moreover, regarding mercury resistance in bacteria, Silver *et al*. (1994) found *Pseudomonas* species to be the predominant isolates from mercury contaminated sites in Minamata. *Pseudomonas* strains also dominated the isolates with the highest resistance to mercury in bioreactors cleaning mercury polluted factory wastewater. *Ps. putida* KT2440 thus appeared to be a promising candidate for the expression of the mercury resistance and GFP.

1.3 Detection and Quantification of Bacteria

In a risk assessment and quite generally in many different contexts of microbiology, bacterial densities need to be determined. In a culture or likewise in an environmental sample the total bacterial density can be quantified, or a subgroup or single strain possessing a specific characteristic can be detected or selected for. This trait may be a specific DNA sequence or a

phenotypic characteristic and it may be natural or introduced. Prerequisite for the detection and/or quantification of a GEM is that it possesses a feature that is unique and absent in the natural community that the organism has been introduced into. Before the rise of molecular techniques, the quantification of microorganisms was based on cultivation in liquid or on solid media, for specific organisms with the application of a selective pressure. With microscopy and the development of DNA staining dyes bacteria could be visualized, however not discriminated. By now a number of cultivation-independent, i.e. molecular-based, methods, have become available and are alternatively used along with cultivation-dependent methods as will be described in the following.

1.3.1 Cultivation-Dependent Detection

Spreading an appropriate dilution of a liquid sample on solid medium for counting colony forming units (cfu) is one of the most classical methods to quantify cell density. It can also be used to detect/quantify cells that exhibit a specific phenotype, e.g. expression of an antibiotic or heavy metal resistance, or the ability to degrade certain compounds. These traits may serve as intrinsic or introduced markers for a particular group of bacteria and the DNA underlying the phenotype, the marker gene, can be defined as "[…] a DNA sequence, introduced into an organism, which confers a distinct genotype or phenotype to enable monitoring in a given environment. An intrinsic marker is a non-introduced DNA sequence or natural phenotype that serves as a signature for a particular organism or group of organisms" (Jansson *et al*. 2000). Examples for genetic markers that can serve as selective markers are resistance to rifampicin (*rif),* kanamycin *(npt*II*),* herbicides (*aro*A*, bar),* mercury *(mer),* arsenic *(ars;* Jansson & Prosser 1997, Prosser 1994).

Many studies investigating bacterial ecology have exploited an intrinsic or introduced marker to monitor the number of colony forming units (cfu) of a particular strain or group of strains on a selective medium. Alternatively, total bacterial densities have been quantified on non-selective medium (e.g. Hill *et al*. 1994, Vionis *et al*. 1998, Leff *et al*. 1998, Lemke & Leff 1999, Kroer *et al*. 1998, Blumenroth & Wagner-Döbler 1998).

The advantages of cultivation are that it is technically easy to manage, inexpensive, and yields bacterial biomass that can be used for further tests or experiments. Selective agar plate counts allow enumeration of colonies arising from individual "viable cells" (Gerhardt 1981).

However, colonies arising from individual or from aggregated cells in pairs or clusters (Johnson 1999) are indistinguishable, so that the cfu per volume may not be equal to the number of cells per volume. In order to discriminate separate colonies it can be necessary to

concentrate the sample or on the contrary to dilute the sample and homogenize well. Soil or sediment samples need to be pre-treated (sonicated or vortexed) to detach cells from particles and transfer them to a liquid phase that can be spread on agar. This treatment will also break up cell clusters. However, if the treatment is too harsh cells may lyse.

A density effect can often be observed at enumeration on selective medium with more numerous cfu than expected at lower dilutions (i.e. higher cell numbers in the sample). This may be due to a concerted action of all resistant cells in the sample eliminating the selective pressure and allowing growth of cells that would normally not be tolerant to the antibiotic or toxin. Cells from environmental samples are required to be culturable with the medium used and be in a physiological state where they are likely to form colonies.

Antibiotics used for selection may be heat- (β-lactams, e.g. ampicillin, or aminoglycosides, e.g. neomycin) or light sensitive (tetracyclin) or may not be soluble in water (thiostrepton) and form suspensions in agar affecting availability and activity (Egan & Wellington 2000). Interactions between the selection marker and the medium constituents need also be considered. Heavy metals such as mercury bind to sulphur-groups of amino acids or proteins. Thus, complex media, such as Luria Bertani (LB) medium with a high proportion of SH-containing amino acids or proteins require higher total amounts of the heavy metal than e.g. a minimal medium in order to yield the same bio-available concentration and effectively repress the growth of non-resistant bacteria (Chang *et al*. 1993, Farrell *et al*. 1993).

For the experiments carried out in this work cultivation provided an easy method to quantify different relevant groups of bacteria within the same sample (GEM, total bacteria, mercury-resistant bacteria). However, because of the drawbacks of the cultivation approach, GEM density was also determined with a cultivation-independent method.

1.3.2 Cultivation-Independent Detection

With the development of molecular techniques it has become possible to detect specific groups of bacteria or individual strains even if cultivation is difficult or impossible: Hybridisation or polymerase chain reaction (PCR) with specific DNA or RNA probes or primers are now standard methods in every ecological laboratory. Primers or probes can be directed against conserved universal sequences, e.g. 16S rDNA, or against marker genes (see above). Bioluminescence is a very comfortable reporter system since it can be detected microscopally during growth in individual cells and does not require cultivation.

1.3.2.1 PCR-based Detection and Analysis

Using molecular approaches it is relatively easy to detect certain bacteria. PCR is a particularly sensitive method: only small amounts of template DNA are required in a polymerase chain reaction to get a good result. Depending on the quality (i.e. purity) of the sample 1-10 ng of bacterial genomic DNA or 0.1-1 ng of plasmid DNA are sufficient (Roche 1999). This makes PCR the method of choice in terms of sensitivity and detection limit.

In combination with high resolution polyacrylamide electrophoresis and a thermal or chemical denaturing gradient (see thermogradient gel electrophoresis, chapter 2.6.4) the molecular approach can be used to monitor the genetic diversity of bacterial communities (Muyzer & Smalla 1998). Identification of single community bands can be achieved by purification of specific bands and sequencing.

The analysis of enzyme encoding genes in a complex community has only just begun. Genes encoding NiFe hydrogenase (Wawer & Muyzer 1995), the large subunit of multicomponent phenol hydroxylase (LmPH; Watanabe *et al*. 1998) or the α- subunit of the methanol dehydrogenase (*mxa*F; Fesefeldt & Gliesche 1997) have been targets for community analysis or detection of bacteria carrying these genes. Recently, the mercury resistant community in the biofilm of a technical scale biocactalyzer has been investigated with *mer*A specific primers (Felske *et al*., submitted). Performance of individual isolates from the effluent could be directly monitored in a *mer*A community profile. This is a fine example how genomic sequence data can be directly correlated with performance. These kind of investigations promise new information on the structure and function of bacterial communities.

In this work the same *mer*A profiling protocol was followed to monitor the mercury-resistant community in a mercury-free and mercury-containing environment.

Using PCR it has also been possible to detect and quantify the *Ps. putida* KT2442::mer73 in bioreactor effluent by exploiting information about the insertion site of the *mer* operon and primer pair design with one primer located within the *mer* operon and one in the flanking genomic sequence of the strain (Felske *et al*. 2001, Felske *et al*. 2002). This method could also be applied in this work for the detection of the GEM.

1.3.2.2 Luciferase Bioluminescence

Luminescence is found mostly in marine animals, but also in insects (firefly). Nevertheless, the most abundant and widely distributed luminescent species are gram negative bacteria (Meighen 1991). The luciferase dependent luminescence involves the enzymatic conversion of a substrate and is dependent on chemical energy. A reduced riboflavin phosphate ($FMNH_2$)

and a long-chain fatty aldehyde are oxidized with the emission of blue-green light (490 nm). The enzyme responsible for the oxidation is called luciferase and its two subunits are encoded by *lux*A and *lux*B of the *lux* operon. Other *lux* genes code for the synthesis of the aldehyde (fatty acid reduction) or possess regulatory functions. The *luxAB* bioluminescence requires reducing equivalents, and is thus directly correlated with the metabolism of the cell representing a good reporter for the metabolic activity of a culture (Unge *et al.* 1999). However, with the decline of the activity of the cell, Lux luminescence ceases. The Lux luminescence has also been used in *mer*R fusions as a reporter for bioavailable mercury in as low as picomolar concentrations (Hansen & Sørensen 2000, Rasmussen *et al*. 2000). *Ps. putida* KT2440 was tagged with Lux bioluminescence for deliberate release into the environment (Ramos *et al*. 2000). Under sterile conditions they found a correlation between inoculum density, light output, and ribosomal contents of *Ps. putida* cells colonizing the root system of barley seedling, proving the system to permit non-destructive *in situ* detection of the strain.

Nevertheless, Lux luminescence dissipates a relatively weak shine in comparison with GFP, and with the auto-fluorescence of *Ps. putida* (and other auto-fluorescent strains in an environmental sample) a strong, high intensity fluorescence is needed to ensure easy detection of the tagged cells.

1.3.2.3 Green Fluorescent Protein

Another form of luminescence is found in the coelenterate *Aequorea victoria*, a jellyfish that lives in the Pacific Ocean and fluoresces dissipating a green shine. The protein responsible for this, the Green Fluorescent Protein (GFP), has been well investigated (Chalfie *et al*. 1994, Yang *et al*. 1996). By energy transfer, it transduces the blue chemiluminescence of another protein, aequorin, into green fluorescent light. The fluorophore originates from an internal Ser-Tyr-Gly sequence, which is post-translationally and non-enzymatically cyclized (to a 4-(*p*-hydroxybenzylidene)-imidazolidin-5-one structure), followed by oxidation of the tyrosine to dehydrotyrosine. This occurs without participation of a co-factor. GFP has been extremely useful as a marker for gene expression and as a tag in protein localization studies in a variety of organisms, including Gram-negative and Gram-positive bacteria and yeast, but also higher organisms such as slime mould, plants, *Drosophila*, zebrafish and mammalian cells.

The GFP wildtype excitation peak is at 395 nm (however, a minor peak appears at 470 nm) and emission is at 508 nm. Due to the secondary peak at 470 nm, wildtype GFP fluorescence can be monitored with a standard fluorescein isothiocyanate (FITC) excitation-emission filter

set. However, the intensity of fluorescence at that excitation wavelength is not optimal. Thus Cormack *et al*. (1996) have isolated mutants that fluoresce more intensely at 488 nm, a wavelength which is congruent with a line of an argon laser that can be used in fluorescence-activated cell sorting (FACS).

A prominent feature of GFP is its high stability. While this enables researchers to observe cells for some while after they have ceased metabolic activity (Lowder *et al*. 2000), the accumulated GFP may become a burden for the cell if present in too high numbers e.g. because it is over-expressed. Furthermore, due to its stability GFP cannot be utilized for transient expression studies. However, Andersen *et al.* (1998) accomplished to tag the C-terminus of the FACS-optimised GFP protein with a short peptide sequence exploiting a natural degradation system (*ssr*A) for proteins resulting from incomplete or damaged mRNA. This peptide tag is recognized by intracellular tail-specific proteases that then rapidly degrade the protein. Andersen *et al*. (1998) developed several destabilized GFP versions by altering the last three residues of the peptide tag yielding GFP proteins with different half lives. Transcriptional fusion of a protein with a destabilized GFP version now allows to investigate real-time *in situ* expression by monitoring the green fluorescence that is lost as expression is halted. Sternberg *et al*. (1999) used these destabilized versions to investigate bacterial growth in biofilms by transcriptional fusion of the *gfp* with a growth-rate regulated, ribosomal *E. coli* promoter. For a review on the application of GFP as reporter of gene transcription and of GFP fusion for protein localization and dynamics see Southward & Surette (2002).

Three different GFP versions of Andersen *et al*. (1998) were also used in this work in the construction of new mercury reducing *Ps. putida* strains. The tagging of these constructs with *gfp* serves GEM control in two ways: it not only allows easy detection of the strain within and outside the bioreactor, but due to a transcriptional fusion of the mercury resistance to *gfp*, the activity of the mercury-reduction and thus performance of the strains can be monitored *in situ*.

1.4 Microcosms

1.4.1 Microcosms are Microscale Ecosystems

Due to the complexity of the processes occurring in nature, imitating the environment or parts of it in artificial systems is always challenging. One of the most fascinating examples for such an enterprise is Biosphere 2 (http://www.bio2.edu/site.htm), a huge glass-house-like structure that was privately built in the late 1980s to discover if eight people could sustain themselves

in a sealed, energy-rich environment. Today, Biosphere 2 has been changed into a research, educational and conference centre being part of Columbia University, Arizona, USA, since 1996. The research includes investigation of physical and geochemical processes, as well as diversity and ecological studies of various habitats from desert to rainforest and ocean. All investigations are dependent on large ecosystem models, so called mesocosms because of their size.

If the ecosystems of interest cannot be studied in the field and without the vastness of Biosphere 2, ecologists must restrict themselves to smaller models that comfortably fit into a standard laboratory. These smaller model ecosystems are called microcosms. A precise definition what makes a microcosm is difficult to phrase due to the versatility of types. Beyers & Odum (1993), have tried to extract what most microcosms have in common, namely all are derived from natural ecosystems, however are no longer in contact with the natural ecosystem. They are compact subsets of the natural system from which they have come and usually contain mixed populations of microorganisms (and/or higher organisms). Most possess spatial heterogeneity at least to some extent and show time-dependent changes in their physical, chemical and biological properties.

Because natural ecosystems encompass innumerable interconnected micro-habitats, a microcosm is well suited to represent certain aspects of a particular habitat, even if lacking integration into a broader ecological context owing to the smaller dimensions.

1.4.2 Ecological Research in Stream Microcosms

Microcosm research aims at understanding the ecology of a particular habitat, and microcosms are often employed to study the effects of a disturbance of a habitat's regular processes. In the context of GEMs and bioremediation, stream environments play a prominent role since wastes are often discharged into rivers where they may affect the biota. Otherwise, if the waste is cleared with a GEM-bioremediation technology, the GEM may be washed into the river and exert an influence on the natural microbial community.

All ecosystems are complex, and this is even more true for rivers which are extraordinarily dynamic habitats: Besides the temporal dynamics owing to seasonal changing (Brümmer *et al*. 2000), streams are characterized by constant spatial (ex)changes, primarily in the horizontal, however to a great extent in vertical direction (Leff *et al*. 1992). Warren & Davis (1971) discussed the difficulties and limitations associated with the modelling of streams. They stated that "as our objectives become more synecological, our laboratory streams and communities must be more like their counterparts in nature". Still, 26 years later, Ashelford *et*

al. (1997) conclude: "[…]. Hence, we require the design of microcosm experiments that more closely mimic what happens in nature".

Several types of stream microcosms have been used for ecological research to study the effect of toxic chemicals (heavy metals, insecticides) or of predation on lotic or benthic macroinvertebrate communities, i.e. those in running waters or living in the river bed (Clements 1999, Jungmann *et al*. 2001, Schulz & Liess 2001). Rarely have stream microcosms been used in microbiology research. However, investigations involving stream bacteria have been carried out in stirred beakers containing water, sediment and leaves (Lemke & Leff 1999, Janakiraman & Leff 1999). Hill *et al*. (1994) used a recirculating stream microcosm (depicted in Ashelford *et al*. 1997) to study the persistence and mobilization of the catabolic recombinant plasmid pD10 in the epilithon.

In this work, a stream microcosm was designed and characterised to be employed in experiments regarding the ecological impact of the mercury reducing GEM *Ps. putida* KT2442::mer73. If the GEM was used in a bioreactor that discharged its effluents into a river the GEM would continuously be liberated in low numbers. A second feasible scenario is the failure of the bioreactor with the release of all its contents into the river, thus supplying shock numbers of GEM cells. The microcosm experiments undertaken in this work pay tribute to these two scenarios by continuous inoculation of the microcosms with the GEM *vs*. inoculation with shock loads in the middle of the experiment.

The liberated microbes would quickly experience dilution by the current, however, some would settle to the sediment, which could serve them as colonization matrix. A supposed influence of the GEM on the indigenous bacterial community would most likely be seen here. Hence, in this work stream microcosms with river sediment were used to study the impact of the GEM.

1.5 Aims of this work

In this thesis the impact of the mercury reducing GEM *Ps. putida* KT2442::mer73 on the native bacterial community in Elbe River water and sediment was investigated in terms of survival and persistence, effect on community composition as depicted by 16S rDNA-TGGE patterns and gene transfer. For this purpose a stream microcosm was designed and characterised regarding its flow. Both, long-term inoculation of the GEM with low cell densities and a cell shock with high densities into the microcosm were investigated. Gene

transfer was studied with and without the presence of a selective pressure in form of phenyl mercuric acetate (PMA).

Although *Ps. putida* KT2442::mer73 was shown to be highly resistant to mercury, it failed to establish good biofilms in laboratory scale bioreactors (von Canstein *et al*. 2002a). Therefore new GEMs were constructed that were selected for high mercury resistance and were in addition provided with green fluorescent protein (GFP). A promoterless cassette consisting of the *mer* operon and *gfp* was inserted into the genome of the fully sequenced safety strain *Ps. putida* KT2440, to set both functions under the transcriptional control of the same host promoter. This way, the GFP tag did not only permit easy detection of the new strains but allowed monitoring of mercury resistance expression via single cell fluorescence. The site of integration was identified to yield genetic information that could be used to develop a highly specific, PCR-based detection method. Furthermore, knowledge of the construct genotype allowed implications regarding the phenotype.

2 Materials and Methods

2.1 Microorganisms and Plasmids

The bacterial strains and plasmids used in this work are listed in Table 2-1.

Table 2-1 Bacterial Strains and Plasmids used in this Work

Strain or Plasmid	Description	Reference
Escherichia coli S17-1/λpir	thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, Km^R, λpir	Miller & Mekalanos, (1988)
Escherichia coli XL1Blue	F'::Tn10, $proA^+B^+$, $lacI^q$, Δ(lacZ)M15, recA1, endA1, gyrA96 (Nal^R), thi, hsoR17 (r_K^- m_K^-) supE44, relA1, lac	Bullock *et al.* (1987)
Escherichia coli JM110	rpsL(Str^R), thr, leu, thi-1,lacY, galK, galT, ara, tonA, tsx, dam, dcm supE44, Δ(lac-ptoAB) [F' traD36 proAB $laq^qZ\Delta M15$]	Yanisch-Perron *et al.* (1985)
Escherichia coli TOP 10	F^-, mcrA, Δ(mrr-hsdRMS-mcrBC), ϕ80, lacZ, ΔM15, ΔlacX74, recA1, deoR, araD139, Δ(ara-leu)7697, galU, galK, rpsL(Str^R), endA1, nupG	Invitrogen, Groningen, The Netherlands
Pseudomonas putida KT2442::mer73	hsdR, $merTPAB^+$, PMA^R, Hg^R, Rif^R, Tol^-, Ben^+	Horn *et al.* (1994)
Pseudomonas putida KT2442::Tc	hsdR, Tc^R, Rif^R, Tol^-, Ben^+	Kindly provided by Niels Kroer[2)]
Pseudomonas putida KT2440	hsdR1, $hsdM^+$, Ben^+	Bagdarisan *et al.*, (1981)
Pseudomonas putida KT2442 ::mer ::gfp	hsdR, $merTPAB^+$, gfp, PMA^R, Hg^R, Rif^R, Tol^-, Ben^+	Kindly provided by Michael Strätz[1)]
Pseudomonas putida KT2440::mergfp41	gfp, hsdR, $merTPAB^+$, PMA^R, Hg^R, Tol^-, Ben^+	this work
Pseudomonas putida KT2440::mergfp46	gfp, hsdR, $merTPAB^+$, PMA^R, Hg^R, Tol^-, Ben^+	this work
Pseudomonas putida KT2440::mergfp47	gfp, hsdR, $merTPAB^+$, PMA^R, Hg^R, Tol^-, Ben^+	this work
pUT-Hg	$oriR6K^+$, $mobRP4^+$, $merTPAB^+$, ΔmerDR, Ap^R, Hg^R	Herrero *et al.*, 1990

pUC19oriT	lacZ (oriT), oriRColE1, Ap^R	Kindly provided by Michael Strätz[1)]
pBluescript II SK+	Derivative of pUC19: lacZ, $oriRColE1^R$, Ap^R	Stratagene, La Jolla, California, USA; Short *et al*. (1988)
pJBA41	$lacZ^{'}$, Ap^R, oriRColE1, gfp($t_{½}$<2min)	Andersen *et al*. (1998)
pJBA46	$lacZ^{'}$, Ap^R, oriRColE1, gfp($t_{½}$=6min)	Andersen *et al*. (1998)
pJBA47	$lacZ^{'}$, Ap^R, oriRColE1, gfp($t_{½}$=36min)	Andersen *et al*. (1998)
pCMGC41	lacZ (oriT), oriRColE1, $merTPAB^+$, Ap^R, Hg^R, gfp($t_{½}$<2min)	this work
pCMGC46	lacZ (oriT), oriRColE1, $merTPAB^+$, Ap^R, Hg^R, gfp($t_{½}$=6min)	this work
pCMGC47	lacZ (oriT), oriRColE1, $merTPAB^+$, Ap^R, Hg^R, gfp($t_{½}$=36min)	this work
pBSL299	oriR R6K, oriT RP4, Ap^R, Sm^R	Alexeyev *et al*. (1995)
pBSL299ΔSm	pBSL299, Sm^R removed by MluI digest and religation	this work
pBVP1	pBSL299ΔSm with gfp from pJBA41	this work
pBVP6	pBSL299ΔSm with gfp from pJBA46	this work
pBVP7	pBSL299ΔSm with gfp from pJBA47	this work

[1)]Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, Germany

[2)]National Environmental Research Institute, Department of Marine Ecology and Microbiology, Roskilde, Denmark

2.2 Cultivation of Microorganisms

2.2.1 Media

The following media were used solid or in liquid form to cultivate bacteria. All media were either autoclaved for 20 min at 121°C, 1 bar vapour pressure or sterilized through a 0.22 µm membrane filter. Carbon sources, mercury, antibiotics, and trace elements that could not be autoclaved were filter sterilized separately and aseptically added to the medium. For solid medium 15 gL^{-1} Bacto Agar was added before autoclaving.

2.2.1.1 Supplements

Media were supplemented with antibiotics or heavy metals to create a selective pressure and prevent contamination during cultivation. The inductor isopropyl-β-D-thiogalactopyranosid (IPTG) was used to induce transcription from the *lac* promoter, 5-bromo-4-chloro-3-indoyl-β-D-galactosid (x-gal) was used in a blue-white screening as a substrate that is metabolised by the intact β-galactosidase yielding bright blue products. If however, the DNA coding for the enzyme (*lac*Z) is disrupted (because a DNA sequence was cloned into the multiple sequence site that lies within the gene), the substrate cannot be metabolised and colonies growing in the presence of x-gal remain white.

For different purposes, different concentrations of mercury in the medium were appropriate. Luria Bertani (LB) medium binds substantial amounts of $HgCl_2$ (see below, Chang *et al.* 1993, Farrell *et al.* 1993), owing to SH-groups present in tryptone and yeast extract (Misra, 1992). Therefore, for the same bioavailable Hg-concentration more mercury needed to be added to LB medium than to a minimal medium. Moreover, *Ps. putida* can tolerate many toxic (organic) compounds to a certain extent without expressing the actual resistance enzyme. This may partially be due to alterations of the outer cell membrane, e.g. mechanisms such as *cis-trans* isomerization (Heipieper 1996). Hence, higher PMA concentrations were appropriate for *Ps. putida* than e.g. for *E. coli*. Cultivation with mercury as selection pressure required lower concentrations than selection of mercury-resistant transformants after a conjugation experiment (see chapter 3.2.4).

Supplements were added to the medium in the following solvents and concentrations (Table 2-2):

Table 2-2 Supplements in Media

Additive	Solvent	Stock Concentration	End Concentration
Ampicillin	70% Ethanol	100 mg ml^{-1}	100 µg ml^{-1}
Kanamycin	dH_2O	60 mg ml^{-1}	60 µg ml^{-1}
Streptomycin	dH_2O	120 mg ml^{-1}	120 µg ml^{-1}
Rifampicin	DMSO	50 mg ml^{-1}	100 µg ml^{-1}
Tetracyclin	dH_2O	5 mg ml^{-1}	10 µg ml^{-1}
$HgCl_2$	dH_2O	10 mg ml^{-1}	as specified
Phenyl mercuric acetate (PMA)	dH_2O	1 mg ml^{-1}	as specified
IPTG	dH_2O	50 mg ml^{-1}	50µg ml^{-1}
x-gal	DMF (Dimethylformamide)	40 mg ml^{-1}	40 µg ml^{-1}

2.2.1.2 Luria Bertani Medium (Sambrook et al. *1989)*

Tryptone and Yeast Extract were obtained from Oxoid (Ltd Basingstoke, Hampshire UK) and Gibco BRL (now Invitrogen- Life Technologies, Karlsruhe, Germany), respectively. Bacto Agar was obtained from Difco Laboratories, Heidelberg, Germany and sodium chloride was purchased from Fluka (Buchs, Switzerland).

Tryptone	10	g		
Yeast Extract	5	g		
NaCl	10	g	17.1	mM
(Bacto Agar	15	g)		
dH_2O	ad 1000 ml			

2.2.1.3 SOC Medium

Tryptone	20.0	g
Yeast Extract	5.0	g
NaCl	0.5	g
dH_2O	ad 980 ml	

After autoclaving, 10 ml of $MgCl_2$ and 10 ml of $MgSO_4$ are added. Prior to use, 2 ml of 20% (w/v) filter-sterilized glucose are added to 100 ml medium.

2.2.1.4 R2A Agar

R2A Agar is recommended by its distributor Fluka for plate counts of water samples using longer incubation periods and was used for plate counts in the Microcosm Experiments. R2A

Agar is available in a ready to dissolve composition and 18.12 g must be suspended in 1 L of distilled water.

Casein acid hydrolysate	0.5	g
Yeast Extract	0.5	g
Proteose Peptone	0.5	g
Dextrose	0.5	g
Starch soluble	0.5	g
Dipotassium phosphate	0.3	g
Magnesium sulfate	0.024	g
Sodium pyruvate	0.3	g
Agar	15	g
dH_2O	ad 1000 ml	

2.2.1.5 M9- Minimal Medium (Sambrook et al. *1989)*

M9-Salt Solution (x10)	100	ml
Trace Element Solution	2.5	ml
dH_2O	ad 1000 ml	

M9 minimal medium was supplemented with 0.2% Na-citrate ($\times 1H_2O$) or 10 mM benzoate as carbon sources.

2.2.1.5.1 M9 Salt Solution (×10)

Na_2HPO_4 x 2 H_2O	89.00 g	0.500	M
KH_2PO_4	29.94 g	0.220	M
NaCl	49.67 g	0.850	M
NH_4Cl	4.01 g	0.075	M
dH_2O	ad 1000 ml		

2.2.1.5.2 Trace Element Solution (×400)

2x Salt Solution	50 ml			
$MgSO_4$	25 ml	6.16	g	0.25 M
$FeSO_4 \times 7H_2O$	2.5 ml	0.25	g	0.018 M
dH_2O	ad 100 ml			

$MgSO_4$ and $FeSO_4$ were prepared separately before addition. $MgSO_4$ was added from a 1 M stock solution and a 10% $FeSO_4$ stock solution was prepared that was titrated to pH 2 by the addition of H_2SO_4. Both stock solutions were sterilized by filtration prior to use.

2.2.1.5.3 Salt Solution (×2)

MgO	0.75	g	0.018	M
$FeSO_4$ x 7 H_2O	2.0	g	0.007	M
$ZnSO_4$ x 7 H_2O	1.44	g	0.005	M
$MnSO_4$ x 4 H_2O	1.12	g	0.007	M
$CuSO_4$ x 5 H_2O	0.25	g	0.001	M
$CoSO_4$ x 7 H_2O	0.28	g	0.0009	M
H_3BO_3 x 7 H_2O	0.06	g	0.0003	M
37% HCl	51.3	ml		
$CHCl_3$	2	ml		
dH_20	ad 1000	ml		

2.2.2 Culture Conditions

Bacteria were cultivated in a suitable medium (see above) and shaken on a rotary shaker overnight for 14-18 h at 180-220 rpm and 30°C (*Ps. putida*) or 37°C (*E. coli*).

2.2.3 Growth Measurements

2.2.3.1 Optical Density

Growth in liquid culture was followed by measuring the optical density at 600 nm with Shimadzu Cell Positioner CPS-260 Spectrophotometer (Shimadzu Deutschland GmbH, Duisburg, Germany) or Ultrospec100 (Pharmacia Biotech, now Amersham Biosciences, Freiburg, Germany).

2.2.3.2 Determination of Cell Density by Cultivation

For the determination of cell density the liquid culture was diluted with 1% NaCl so that colony numbers on solid medium (petri dish, 16 cm in diameter) could suitably be counted (between 30 and 300 colonies per plate). Serial dilutions of the liquid culture were prepared of which three successive dilutions were plated in triplicates to validate the results (see also 2.8.6).

2.3 Deoxyribonucleic Acid (DNA) Extractions

2.3.1 Mini-scale Plasmid Preparation (Alkaline Lysis Method)

For a mini-scale plasmid preparation 1.5 ml of an overnight grown *E. coli* culture were centrifuged in a microcentrifuge (1 min at 10000×g) and the pellet resuspended in 100 µl resuspension buffer. Then, 200 µl lysis buffer were added and the mixture carefully mixed by inverting the tube. At last, 150 µl neutralisation buffer were added, the tube was inverted a few times, and the preparation centrifuged for 5 min at 10000×g (see 2.3.1.1 for composition of resuspension-, lysis- and neutralisation- buffer). The aqueous phase was removed and transferred to a microcentrifuge tube, and the plasmid DNA precipitated with 1 ml of absolute ethanol and 50 µl of 3 M Na-acetate (pH 5.8). Immediately after adding ethanol and acetate the tube was centrifuged for 30 min at 10000×g, the resulting DNA pellet was washed with 70% ethanol and finally the dried pellet resuspended in 100 µl dH_2O. This method also retrieves RNA which usually does not interfere with the DNA on an agarose gel. If the plasmid was digested with restriction enzyme(s) for analysis, RNAse was added. One to three µl of plasmid were visualized on an 0.8% agarose gel to estimate the concentration of the retrieved DNA. Plasmids were stored at –20°C.

2.3.1.1 Alkaline Lysis Buffers

Re-suspension Buffer (AlkLysI)

Glucose	50	mM
Ethylene diamine tetraacetic acid (EDTA; pH 8.0)	10	mM
Tris/HCl (pH 8.0)	25	mM

Glucose was added at 9 g L^{-1}. Tris/Cl was diluted from a 1 M (219.1 g L^{-1}) stock solution of Tris(hydroxymethyl)aminomethane phosphate that had been adjusted with HCl to pH 8.0. EDTA was diluted from a 0.5 M (167.6 g L^{-1}) stock solution of disodium EDTA that was adjusted to pH 8.0 with HCl.

Lysis Buffer (AlkLysII)

NaOH	0.2	M
SDS	1%	

Neutralization Buffer (AlkLysIII)

K-Acetate (5M)	60	ml
Glacial acid	11.5	ml
dH_2O	ad 100	ml

2.3.2 Preparation of Genomic DNA from Bacteria

2.3.2.1 From Overnight Culture- Method 1 (Asubel et al*. 1990)*

Of a well grown overnight bacterial culture 1.5 ml were centrifuged (1 min at 10000×g) and the bacterial pellet resuspended in 567 µl TE buffer. Moreover, 30 µl of 20 mg ml^{-1} proteinaseK (to give a final concentration of 100 µg ml^{-1}) and 3 µl of 10% SDS (to give a final concentration of 0.5%) were added. The mixture was incubated for 1 h at 37°C and 100 µl of 5 M NaCl added. Polysaccharides were complexed by addition of 80 µl hexa-decyl-trimethyl ammonium bromide (CTAB) and 10 min incubation at 65°C. Proteins were removed by double chloroform/isoamyl alcohol (24:1) extraction (see also 2.4.4.2). The aqueous phase was transferred into a fresh microcentrifuge tube and the DNA precipitated with 0.6 vol. isopropanol, washed with 70% ethanol and the dry pellet resuspended in 50 µl dH_2O or TE-buffer.

TE-Buffer

Tris/Cl (pH 8.0)	10	mM
EDTA (pH 8.0)	1	mM

CTAB/NaCl Solution

NaCl	4.1	g
CTAB	10	g
dH_2O	ad 100	ml

NaCl was dissolved in 80 ml of dH_2O and the CTAB slowly added while heating and stirring. Once the CTAB was fully dissolved, the volume was adjusted to 100 ml.

2.3.2.2 From Overnight Culture- Method 2

Alternatively, genomic DNA from pure bacterial cultures was isolated with the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions:

One ml of an overnight LB culture of the desired strain was centrifuged for 10 min at 6000×g in an Eppendorf microcentrifuge 5415C (Eppendorf AG, Hamburg, Germany), the supernatant removed and discarded. The pellet was resuspended in buffer T1 by pipetting up and down. From a stock solution 0.6 mg of Proteinase K was added, the contents of the reaction vessel mixed by vortexing and incubated at 56°C with occasional vortexing. When lysis was completed and the suspension was clear (usually after 20 min) 200 µl of buffer B3 were added to the sample and the mixture incubated for an additional 10 min at 56°C. After the addition of 210 µl of 98% (v/v) ethanol the sample was vortexed, loaded into a NucleoSpin Tissue column and centrifuged for 1 min and 8000×g in a microcentrifuge. The flow-through was discarded and the matrix-bound DNA washed first with 500 µl of BW buffer followed by a double washing step with 500 µl of B5 buffer. Finally, the column was centrifuged for 2 min at 10000×g to remove residual ethanol, the column transferred into a fresh 1.5 ml reaction tube and the DNA eluted in a microcentrifuge at 10000×g with 200 µl of BE buffer, that had been pre-heated to 70°C.

2.3.2.3 From Sediment Samples

Total DNA from soil was extracted using the Fast DNA Kit for Soil (Bio101, Inc., Carlsbad, CA, U.S.A) and following the instructions of the manufacturer: The sediment (0.5 g) was added to a Multimix 2 Tissue Matrix Tube that contained ceramic and silica beads. Sodium Phosphate Buffer (978 µl) and MT Buffer (122 µl) were added and the tube processed for 2 min in a bead beater MM2000 (Retsch GmbH & Co. KG, Haan, Germany). After homogenisation the tube was centrifuged at 10000×g for 30 sec. The supernatant was transferred to a clean tube and 250 µl PPS reagent were added. After mixing the sample carefully by inverting the tube by hand about ten times the tube was centrifuged at 10000×g for 5 min to pellet the precipitate. The supernatant was then transferred to a clean 15 ml tube and 1 ml of binding matrix was added. The mixture was inverted by hand for 2 min and placed back into the rack to allow settling of the silica matrix. Of the supernatant 500 µl were carefully removed and the silica matrix resuspended in the remaining supernatant. The suspension was transferred to a spin filter and centrifuged at 10000×g for 1 min in portions of 600 µl until everything had passed the filter. The filter column was washed with 500 µl of SEWS-M Buffer and the column centrifuged for an additional 2 min to dry the matrix. The column was then air-dried by leaving it on the bench for 5 min at room temperature and the DNA eluted with 50 µl DNase/Pyrogen free water provided by the manufacturer.

2.3.2.4 From Water Samples

Water samples (10 ml) from the microcosms (2.8.5) were complemented with 0.1 volume of sodium acetate and 2.5 volumes of absolute ethanol and kept at –20 °C until further use. For DNA extraction samples were centrifuged for 30 min at 20000×g, (Sorvall R5C5, SA-600 Rotor). The supernatant was discarded and the pellet resuspended in 750 µl TE Buffer. The DNA was then loaded to a NucleoSpin column from the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) and washed twice with B5 Buffer from the same kit. The column was centrifuged again to remove residual wash buffer and the DNA eluted with 100 µl BE buffer (Macherey-Nagel, Düren, Germany) that had been heated to 70°C.

2.3.3 Plasmid Preparation and Purification

For quick mini scale or maxi scale plasmid preparations, plasmid kits from QIAGEN (AG, Hilden, Germany) were used: After pelleting 1 ml of an overnight bacterial culture, cells were resuspended in a microcentrifuge tube in 250 µl of P1 Buffer. For cell lysis 250 µl of P2 Buffer were added and the tube inverted 4-6 times. pH neutralization and precipitation of chromosomal DNA and cell components was achieved with addition of 350 µl of N3 Buffer. After inverting the tube 4-6 times the solution had become cloudy and was centrifuged for 10 min at 10000×g. The supernatant was then applied to a QIAprep spin column. The column was centrifuged for 30 sec at 10000×g, the flow-through discarded, and the spin column washed to remove trace nuclease activity by adding 0.5 ml of PB Buffer and centrifuging for 30 sec at 10000×g. After removal of the flow-through, the column was washed with 0.75 ml of PE Buffer and centrifuged for 30 sec at 10000×g and the flow-through discarded. For removal of residual wash buffer, the spin column was centrifuged for an additional 1 min, and the DNA eluted with 50 µl of sterile dH_2O.

2.4 DNA Standard Techniques

2.4.1 DNA Gel Electrophoresis

DNA fragments can be separated according to size (length and/or conformation) if loaded on an agarose gel and voltage is applied. Fragments move faster if the agarose concentration is low (e.g. 0.8%) which favours the visualization of larger fragments and slower if the concentration is great (e.g. 2%) which favours the visualization of smaller fragments. The agarose is added to 1×Tris-Acetate-EDTA (TAE)-Buffer (Sambrook *et al.* 1989) to give a

suitable concentration, melted by boiling, and cooled to ca. 55°C at which it is still viscous and can easily be poured into an electrophoresis chamber (Horizontal Gel Electrophoresis Apparatus, GIBCO BRL Life Technologies). After the gel had set, it was covered with 1×TAE Buffer, loading dye was added to the DNA samples. Then, the DNA samples and a DNA size marker were loaded onto the gel and a voltage of 80-100 V was applied (BioRad, Model 200/2.0 Power Supply). The loading dye is necessary for the DNA sample to settle in the pocket, it also indicates how far the DNA fragments have travelled inside the gel by showing coloured bands. After electrophoresis, the gel was immersed in an ethidium bromide bath (1µg ml^{-1} dH_2O) for 10 to 15 min and washed in dH_2O. DNA can be stained with ethidium bromide which intercalates with the DNA double helix and can be made visible with UV light (wavelength 245 nm). Gels were illuminated with a UV Transilluminator (San Gabriel, USA, Model M-26-E), photographed with a CCD-camera (Herolab Model 429K) and manipulated with analysis software E.A.S.Y. from Herolab, Wiesloch. Processing of the photographs was accomplished with a video printer (Mitsubishi Video Copy Processor).

TAE-Buffer (1×)

Tris-Acetate (pH 7.5)	40	mM
EDTA (pH 8.0)	1	mM

Loading Dye

Sucrose	40%	(w/v)
Bromophenol blue	0.25%	(w/v)
Xylenecyanol FF	0.25%	(w/v)
EDTA (pH8.0)	0.1	M

2.4.1.1 DNA Size Markers

Following DNA size markers were used to determine DNA fragment length. DNA markers were purchased from Boehringer Mannheim (now Roche Biochemicals, Table 2-3 DNA standards [in kb]).

Table 2-3 DNA standards [in kb]

DNA Molecular Weight Marker X[1)]	DNA Molecular Weight Marker III (DIG-labeled)[2)]	DNA Molecular Weight Marker XIV (100 bp ladder) [3)]
12.216	23.13	2.642
11.198	9.42	1.500
10.180	6.56	1.400
9.162	4.36	1.300
8.114	2.32	1.200
7.126	2.03	1.100
6.108	0.56	1.000
5.090	0.13	0.900
4.072		0.800
3.054		0.700
2.036		0.600
1.635		0.500
1.018		0.400
0.517		0.300
0.506		0.200
0.396		0.100
0.344		
0.298		
0.220		
0.201		
0.154		
0.134		

[1)] Mixture of a 1018 bp fragment and its multimers and pBR322 fragments (23 fragments)

[2)] λ-DNA, cleaved with HindIII (8 fragments)

[3)] Enzyme pattern of specifically constructed enzyme (15 fragments)

2.4.2 DNA Quantification

Boehringer Mannheim (now Roche) provides λ phage DNA solutions of defined concentrations (62.5, 125. 250, 500 ng/6 µl) which are useful for a rough estimation of the sample DNA concentration from the ethidium bromide signal in the agarose gel. For more exact measurements, the absorption at 260 nm was determined in a spectrophotometer. An absorption of 1 equals 50 µg dsDNA ml^{-1} (Sambrook *et al.* 1989). Protein contamination could be taken into consideration by taking an additional absorption measurement at 280 nm. The ratio [absorption at 260 nm/ absorption at 280 nm] should be approximately 1.8 - 1.9 for pure DNA (Sambrook *et al.* 1989).

2.4.3 Enzymatic DNA modification

2.4.3.1 Restriction Enzyme Cleavage

Restriction digests were performed with restriction enzymes from New England Biolabs (NEB, Inc., Beverly, Maine, USA), with the appropriate 1× buffer for 2 h, at the temperature that was recommended for a particular enzyme by the manufacturer. Incubations were carried out in a thermoblock or waterbath, and Bovine Serum Albumin (BSA) was added if recommended by the manufacturer. Enzyme concentration was chosen in agreement with the recommendations of NEB depending on DNA and enzyme stock concentration, adding 1 µl of enzyme to 50 µl restriction digest mix.

2.4.3.2 DNA Dephosphorylation

In the progress of cloning, insert and vector may be ligated after having been digested with the same restriction enzyme(s). If digested with a single restriction enzyme only, the vector can recircularise during ligation without incorporating an insert fragment, and thus reducing cloning efficiency by increasing the vector background. This can be avoided if the vector is dephosphorylated, i.e. if the DNA 5'-phosphoryl termini required by ligases are removed. Dephosphorylation was achieved by treatment with Calf Intestine Alkaline Phosphatase (CIP, Boehringer Mannheim, now Roche, Mannheim, Germany), during which 1 pmol 5' phosphorylated (sticky or blunt end) DNA were incubated with 1 unit of Alkaline Phosphatase for 1 h at 37°C.

2.4.3.3 Fill-In of 3' Recessed Ends

The Large Fragment (Klenow Fragment) of *E. coli* DNA Polymerase I possesses the ability for polymerisations but in contrast to the holoenzyme does not degrade 5' termini. It was used for filling in 3' recessed ends, i.e. making blunt ends from sticky ends, if no suitable restriction site was to be found for a cloning strategy. If a particular orientation of the insert within the plasmid was desired, restriction digest or PCR needed to be performed for analysis of the plasmid.

2.4.3.4 Ligation

For genetic manipulations it was commonly necessary to insert a fragment into a particular plasmid vector. To achieve this, the circular plasmid vector was cut open enzymatically with either the same endonuclease that had been used to prepare the insert, or with an enzyme that resulted in compatible overhangs. Plasmid and vector then needed to be ligated to be

covalently bonded. The enzyme T4 DNA ligase catalyses the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hyroxyl termini in duplex DNA or RNA in the presence of ATP. It can join both, blunt-end and cohesive end termini and repair single stranded nicks in duplex DNA or RNA. For a ligation a surplus of the insert was supplied exceeding the plasmid vector by 3–10 times. The 10 µl reaction volume was made up of 1 µl of 10×ATP-containing reaction buffer, 1 µl of T4 DNA ligase (New England Biolabs GmbH, Frankfurt/Main, Germany), between 5 and 15 ng of plasmid vector and between 45 and 100 ng of insert. If the total volume of 10 µl was not reached, the corresponding amount of dH_2O was also added to the mixture. The ligation mixture was incubated over night at 14 °C and plasmids transformed the next day (see 2.4.5).

2.4.4 DNA Purification Techniques

For most molecular applications pure DNA is required as contamination may have inhibitory effects on enzymatic reactions. Hence, the removal of RNA, proteins or salts is mandatory.

2.4.4.1 RNA Removal

Plasmid DNA isolated with the Alkaline Lysis Method was usually contaminated with RNA which could be fully removed if 1 µl of 100 µg ml^{-1} RNAse (Bovine Pancreas, Boehringer Mannheim, now Roche, Mannheim, Germany) was added to a 30 or 50 µl restriction digest.

2.4.4.2 Phenol/Chloroform Extraction

Preparations of chromosomal DNA could not be purified in spin columns due to the size of the genome. However, it could be purged of protein by chloroform/isoamyl alcohol (24:1 (v/v)) extraction. For very pure DNA, a phenol/chloroform/isoamyl alcohol (25:24:1) extraction was performed. Phenol removes proteins more powerfully but it interferes with many molecular methods (e.g. inhibits PCR if present in concentrations >0.2%; QIAGEN News Letter, no.1, 1997). Therefore, if phenol extractions could not be avoided they were always succeeded by chloroform extraction by which most of the phenol was removed. Traces of the volatile chloroform disappeared if the DNA was vacuum dried (DNA Speed Vac-DNA120 SAVANT SS1, Savant Instruments, Farmingdale NY, USA).

2.4.4.3 DNA Precipitation

The DNA was precipitated with 0.1 volume 3 M Na-acetate (pH 5.8) and 2.5 volumes of absolute ethanol. It was usually sufficient to centrifuge the DNA immediately after addition of acetate and ethanol at least 15 min at 10000×g (microcentrifuge). The DNA pellet was

washed with 70% ethanol, dried and resuspended in an appropriate volume of dH_2O or Tris-HCl buffer (50 or 100 µl).

2.4.4.4 DNA Extraction from Agarose Gels

QIAGEN provides systems for quick and easy DNA purification for DNA between approximately 70 bp and 10 kb in size. A maximum of 10 µg can be recovered with that method. The DNA is added to a spin column which is centrifuged to bind the DNA to the silica-gel membrane. The DNA is then washed with PE Buffer and eluted with distilled water or Tris/Cl Buffer. In this work, the QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit were used and the instruction manual followed. Specific DNA bands were extracted and purified from agarose gel in TAE buffer with the QIAquick Gel Extraction Kit from QIAGEN (AG, Hilden, Germany). DNA bands were excised with a scalpel and treated further according to the instruction manual. The excised gel slices were weighed and 3 volumes of QG Buffer were added (e.g. 300 µl to 100 mg of gel). They were then incubated at 50°C for 10 min with occasional vortexing. According to the manufacturer's instruction 1 gel volume of isopropanol was added to the sample if the DNA fragment was <500 bp or >4 kb. Next, a QIAquick spin column was placed into a 2 ml collection tube, the sample loaded to the column and centrifuged for 30 sec at 10000×g for 1 min. After removal of the flow-through, the column was washed with 0.75 ml PE Buffer, centrifuged for 30 sec at 10000×g, the flow-through was discarded and the column centrifuged for an additional 1 min at 10000×g. The DNA was eluted with 30 or 50 µl of sterile dH_2O.

2.4.4.5 Purification of DNA in solution

For purification of DNA-solutions (e.g. of PCR products), the liquid DNA sample was mixed with 5 sample volumes of PB Buffer (e.g. 500 µl of PB Buffer to 100 µl of DNA sample) and loaded to a QIAquick spin column. Purification was then carried out as explained in 2.4.4.4 for DNA extraction from gel slices.

2.4.5 Introduction of Plasmids into Bacterial Cells

2.4.5.1 Transformation by Electroporation (Dower et al. 1988)

Under specific conditions some bacteria have the ability to take up naked DNA. In this state they are described as being competent. In the laboratory this phenomenon is exploited to introduce plasmids into bacterial cells. Standard organism for transformation is *E. coli*, however, other organisms, such as *Pseudomonas*, can be electroporated (Artiguenave *et al*.

1997). Electroporation is a very efficient method of transformation during which a current is applied to a mixture of cells and plasmid molecules. This generates pores in the cell membrane through which the plasmids can enter the cell. Electro-competent cells (*E. coli* XL1Blue) were obtained from Stratagene. Before electroporation 6 µl of a dialyzed ligation were added to 20 µl of electro-competent cells in a 0.5 ml reaction tube and incubated on ice for ca. 2-3 min. The mixture was then transferred into an electroporation cuvette (2 mm, EQUIBIO) and electroporated. For Electroporation the BioRad Gene Pulser was used at an extender capacitance of 500 µFD, a resistance of 200 Ω, a pulse controller capacitance of 25 µFD, and a voltage of 2.5 V. Standard time constant for electroporation was 4.5 msec. Immediately after the electroporation, the cells were resuspended in 1 ml of LB medium and incubated for 1 h at 37°C. Thirty, 80 and 150 µl of the cells were then plated on selective LB medium.

To obtain electro-competent cells an LB culture of *E. coli* S17-1/λpir was grown to mid-log phase (OD_{600}= 0.8 - 1.0) and chilled on ice for 30 min before harvest (10 min, 4°C, 4000×g, Sorvall RC5C). The pellet was washed twice with 1 volume of ice cold water and twice with 1 volume of ice cold 10% (v/v) glycerol. At last cells were resuspended in 1/100 volume of ice cold 10%(v/v) glycerol, divided into 40 µl aliquots and stored at -70°C until use.

2.4.5.2 Chemical Transformation (Stratagene)

For the construction of mercury-reducing fluorescent *Pseudomonas* strains, the restriction enzyme *Bcl*I was used to open the DNA directly downstream *mer* and insert *gfp* (also see 3.2.3). However, in most *E. coli* strains, the *Bcl*I site is methylated by the Dam-methylase and cannot be recognised by the enzyme. Therefore, the Dam^- *E. coli* strain JM110 that permits restriction was used for the cloning. *E. coli* JM110 was obtained from Stratagene (La Jolla, California, USA) and transformed according to the following protocol: The competent cells that had been stored at -70°C were thawed on ice and carefully mixed by hand. Into a pre-chilled 15 ml Falcon tube 100 µl of the cells were then transferred and complemented with 1.7 µl of β-mercaptoethanol to give a final concentration of 25 mM. The mixture was carefully swirled and incubated on ice for 10 min, gently swirled every 2 min. Approximately 50 ng of plasmid was added and the tube incubated on ice for 30 min. A heat pulse was applied for 45 sec in a pre-warmed 42°C water bath after which 0.9 ml SOC medium were added immediately and the transformed cells incubated for 1 h at 37°C on a shaker (200 rpm). The heat-pulse transformed cells were selected by spreading 200 µl on LB solid medium containing ampicillin and incubating at 37°C overnight.

E. coli JM110 was rendered competent according to the Hanahan protocol (Hanahan 1983) yielding ≥5x10^6 transformants per µg DNA according to Stratagene. Transformation efficiency was tested by adding 1 µl of control plasmid (pUC18, 100 pg µl^{-1}) to the competent cells and following above protocol. Plating 200 µl on LB agar medium yielded 36 transformant colonies which calculates to a transformation efficiency of about one third of the efficiency stated by Stratagene.

2.4.5.3 Conjugation (Mating Experiments)

Certain plasmids can also be transferred from one to another cell of the same or another strain by conjugation. This requires proteins involved in the plasmid transfer, so called *tra*-functions, and the plasmid to contain an origin of transfer (*ori*T) at which transfer is initiated. The strain harbouring the plasmid of interest is termed donor, the one to receive the plasmid recipient. If the donor possesses all transfer functions necessary, a biparental mating can be performed. In a triparental mating a third strain is needed (helper) that contains a plasmid carrying the *tra*-functions which must first be transferred to the donor for it to be able to transfer the plasmid of interest to the recipient. *E. coli* S17-1λpir possesses all functions necessary for the transfer of a plasmid and is thus a candidate to be used as a donor in a biparental mating. For the mating experiment donor and recipient (*Ps. putida* KT2440) were grown to mid log phase (optical density of 1 at 600 nm) in selective medium (LB medium with ampicillin and kanamycin or ampicillin only). Of these cultures 1.5 ml were spun down respectively and washed twice in LB medium. The donor and recipient pellet were resuspended together in 30 µl LB medium and transferred to a mating filter (MF-Millipore Membrane Filter, 0.45 µm pore size; Millipore GmbH, Eschborn, Germany) which was incubated overnight at 37°C. The filters were then inserted into a 1.5 ml reaction tube and 500 µl of M9 medium or 1% NaCl added. The tube was vortexed to wash the cells off the filter and 50, 100, and 200 µl plated on selective plates.

2.4.5.4 Minitransposons

Minitransposons present a special case of conjugative plasmids as they are not intended to be replicated in the recipient. Rather, a gene of interest, for this work a *mer-gfp* cassette is to be transposed into the genome of the recipient. Genomic localization of the cassette also serves a prerequisite in the construction of the mercury-reducing, fluorescent microbes that would possibly be used in bioremediation, i.e. a minimized chance of gene-transfer. While plasmids are relatively easily transferred horizontally, transposition renders stable constructs with chromosomally integrated genes if the enzyme responsible for the integration, the

transposase, is not transferred in the course. Minitransposons like the pUT vector (de Lorenzo *et al*. 1990, Herrero *et al*. 1990) or its derivative pBSL299 that suitably possesses a multi cloning site (Alexeyev *et al.* 1995), have been developed for such purposes. The mini-transposon vectors carry an antibiotic-resistance gene outside the 19 bp insertion sequences (IS50) that is not transferred in the transposition and can be used as selection marker during the cloning. The insertion sequences, also called inner (I)- and outer (O)-ends, are flanking the gene that is to be integrated. Most of the pUT- or pBSL mini-Tn5 transposon variants carry an additional antibiotic resistance gene between I- and O-end for better selection of the transformants later on. The pUT- or pBSL mini–Tn5 is replicated from a π-dependent origin of replication, the *oriR* of the plasmid R6K. Stable replication from this origin requires the interaction with the *pir*-protein, that can be provided *in trans* only by some bacterial strains such as *E. coli* S17-1/λpir (Table 2-1). Conjugal transfer is initiated at the origin of transfer, the *oriT* of the plasmid RP4.

2.4.6 Polymerase Chain Reaction (PCR)

PCR is an automated *in vitro* method that allows rapid amplification of a DNA sequence lying between two regions of known DNA. PCR requires a thermostable polymerase (in this work Taq DNA Polymerase from Qiagen, Hilden, Germany), short DNA starting molecules (oligonucleotide primers, usually synthesized by Gibco BRL, now Invitrogen, Karlsruhe, Germany), 2.5 mM of each deoxynucleotide triphosphate (dNTP; MBI Fermentas, St. Leon-Rot, Germany), and suitable reaction conditions (e.g. the 1× Qiagen PCR Buffer contains 1.5 mM MgCl. Mg^{2+} cations are required by the polymerase). DMSO stabilizes single stranded DNA and was added up to 5% if the PCR did not yield any product. Qiagen offers a PCR enhancer (Q-solution) which increases the product yield and was sometimes added in the place of DMSO. PCR was performed in an Eppendorf Mastercycler®Personal (Hamburg, Germany). In the first PCR step the double stranded DNA is melted at 94°C (heat denaturation). In the second step (annealing), the primers are given an opportunity to anneal with the template DNA. The optimal temperature for this step depends on the length and base composition of the specific primers. Strand synthesis occurrs during the last step (extension) and is carried out at a temperature optimal for the enzyme (68°C). This cycle was repeated 24-29 times, which was sufficient to produce ample DNA of at least 5 ng μl^{-1}. A final extension step of 68°C three times as long as the normal extension was apended to ensure that strand synthesis be completed for all products. The success of the PCR was verified on an

0.8% agarose gel. The oligonucleotide primers used in this work and their annealing temperatures can be found in Table 2-4.

2.4.7 Inverse Polymerase Chain Reaction (IPCR)

Ps. putida KT2442::mer73 carries a *mer* operon randomly integrated into the chromosome by transposon mutagenesis. However, the exact location of the insertion had to be revealed. IPCR and subsequent sequencing were performed in order to elucidate the DNA sequence flanking a region of known DNA (Figure 3-21, Ochman *et al*. 1988).

Approximately 1 µg of *Ps. putida* KT2442::mer73 chromosomal DNA was digested with 12 different restriction enzymes, respectively (*Aat*II, *Apa*I, *Bam*HI, *Dra*I, *Kpn*I, *Not*I, *Sma*I, *Spe*I, *Ssp*I, *Pst*I, *Xba*I, *Xho*I). In order to reveal the size of the fragment carrying the *mer operon* a Southern Blot (see 2.5) was performed. The ligation was carried out overnight at 14°C with T4 Ligase (New England Biolabs GmbH, Frankfurt/Main, Germany) and ca. 50 ng (0.05 vol) of the digested chromosome. Of the ligation 1 µl was used as template for the PCR [5 µl 10× Buffer, 5 µl Q-solution, 5 µl of each 10 µM primer (SELLA&NARD, see Table 2-4), 5 µl of dNTPs (2.5 mM each), 0.3 µl Taq Polymerase (5 U μl^{-1}), ad 50 µl with sterile dH_2O]. Enzyme, buffer and Q-solution were purchased from Qiagen, Hilden, Germany. The dNTPs were purchased from MBI Fermentas (St. Leon-Rot, Germany) and diluted to give a concentration of 2.5 mM each. The PCR was repeated with nested primers (TSRIF&LAIRT, Table 2-4) and the signals compared. The bands with the correct difference in size were extracted (2.5.3) and sequenced. Performance of IPCR required the ligation of the fragment to join the unknown ends of the DNA sequence to a circle. Primers were designed to anneal to the ends of the known sequence but to allow “outward” amplification (Table 2-4, Figure 3-21).

Table 2-4 Oligonucleotide Primers

Primer	Sequence (5' - 3')	Annealing temperature	Product /-Length
ALLES	AAGCTTAAGCTATTCCAATCCAG	64°C	forward and reverse primers for detection of *mer*TPAB [3.1 kb]
DRAN	GATATCCGATCACGGTGTCC	66°C	
SELLA	CTGGATTGGATAGCTTAAGCTT	64°C	inverse primers to ALLES/DRAN
NARD	GGACACCGTGATCGGATATC	66°C	
FIRST	GGATCGGCAACTTGACG	54°C	nested forward and reverse primers for detection of *mer*TPAB [2.8 kb]
TRIAL	GCAAAGAAATGTACATGG	50°C	
TSRIF	CGTCAAGTTGCCGATCC	54°C	inverse primers to FIRST/TRIAL
LAIRT	CCATGTACATTTCTTTGC	50°C	
8062for	AGTGTGGTCAGCAACTGGTT	60°C	chromosomal DNA of *Ps. putida* KT2442::mer73 flanking the *mer*TPAB integration site, 2032 bp up-and 924 bp down-stream
7829rev	AACATGGCAATCACATGCCA	58°C	
UPfor	TGAAGTCACCGTAGGCAT	52°C	chromosomal DNA of *Ps. putida* KT2442::mer73 flanking the *mer*TPAB integration site, 338 bp up-and 136 bp down-stream
DOWNrev	TGCTCGTGATAAGTGGACA	53°C	
GFPfor	TGAATTAGATGGTGATGT	48°C	forward and reverse primers for detection of *gfp* [655 bp]
GFPrev	ATCCATGCCATGTGTAATC	54°C	
GFPrev-inv	GATTACACATGGCATGGAT	54°C	Inverse primer to GFPrev
U968-GC1	CGCCCGGGGCGCGCCCCGGGC GGGGCGGGGGCACGGGGGG-AACGCGAAGAACCTTAC	54°C	forward and reverse primers for 16S rDNA amplification and GC clamp for TGGE (Engelen *et al*. 1998)
R-1401	CGGTGTGTACAAGGCCC		
MUTX	CTGTTGCAGACCATGTCATCTAG GAACCGTGATCGGATCGGCCGA GGCGGCCAGATC	54°C	primers introducing a point mutation (G) to the competitive PCR standard (Felske *et al*. 2001)
MUTp	CGGGGAACACGCAGATCAGCCG TTCCTTGC		
PROb	CAGACCATGTCATCTAGGAC	54°C	amplification of competitive PCR standard template (Felske *et al*. 2001)
PROp	CGCAGATCAGCCGTTCCTTGC		
RECb	CATCTAGGACACCGTGATC	54°C	primer used for competitive PCR (Felske *et al*. 2001) [180 bp]
GC-RECp	CGCCCCCGCCGCCCCGCCGCCC GCCGCCCCGCCCCCGCCCAGCC GTTCCTTGCCTTTAC		

GC-merAfor	CCCGCCGCCCCGCCCGCCGCCC CGCCCCGCCGCCCGCCTTGGAC AACGTGC	gradient 36 - 51°C	forward and reverse primers for detection of *mer*A (Felske *et al*. submitted) [280 bp]
merArev	ACGTCCTTGGTGAAGGTCTG		

2.5 Southern Blotting

Southern Blots were prepared before IPCR to determine the size of the *mer* operon carrying fragment (see 2.4.7).

2.5.1 DNA Labelling

In order to obtain a template for the generation of DNA probes PCR was performed with primers annealing respectively to the 5' end and 3' end of the *mer* operon. The product was separated by agarose gel electrophoresis (0.8% agarose) and the 3.1 kb-*mer*-fragment excised. For labelling of the mer DNA probe, Boehringer Mannheim (now Roche, Mannheim, Germany) DIG DNA Labelling Kit was used. Approximately 50 ng of the fragment were denatured in a boiling water bath and chilled quickly on ice/NaCl. Hexanucleotide Mix (2 µl), dNTP-Mix (2 µl), 1 µl Klenow enzyme, and sterile water were added to give a final volume of 19 µl. The mixture was centrifuged briefly and incubated overnight at 37°C. The reaction was stopped after 14 h through addition of 2 µl 0.2 M EDTA (pH 8.0) and the DNA precipitated 2 h at –20°C with 2.5 µl 4 M LiCl and 75 µl pre-chilled absolute ethanol. The DNA was then centrifuged for 15 min at 10000×g and the pellet washed with 50 µl cold 70% ethanol. The dried pellet was dissolved in 50 µl TE buffer.

2.5.2 Southern Transfer

In order to determine the size of the fragment carrying the mer operon, about 2 µg chromosomal DNA of *Ps. putida* KT2442::mer73 were digested with twelve restriction enzymes respectively. The fragments were separated in an 0.8% agarose gel (DIG-labelled size marker, see below), stained with ethidium bromide, and quickly photographed under UV-light for documentation to avoid DNA damage by long exposure of the gel to UV light. The gel was cross linked (UV-Stratalinker 1800, Stratagene, La Jolla, USA) for 2 min. This favours the transfer by introducing strand nicks. It was washed 20 min in 0.2 N HCl to depurinate the DNA, washed in dH_2O three times for 1 min, afterwards twice Denaturation Buffer for 15 min, and then once in Neutralization Buffer for 30 min. During the washes, the nylon membrane (PALL, positively charged nylon membrane Biodyne plus ZNXGR, pore

size 0.45 µm) and Whatman paper were cut to match the size of the gel (11 x 14 cm) and four sheets of Whatman paper soaked in 20xSSC. From bottom to top one dry sheet of Whatman paper, membrane, gel and four sheets of the soaked Whatman paper were laid in the vacuum dryer (BioRad Model 583 Gel Dryer, Bio-Rad Laboratories GmbH, München, Germany) and vacuum applied for 2 h. After the transfer, the membrane was cross-linked and washed with dH_2O.

Denaturation Buffer

NaCl	87	g	1.5 M
NaOH	20	g	0.5 M
dH_2O	ad		1000 ml

Neutralization Buffer

Tris Base	121.1	g	1 M
NaCl	116	g	2 M
dH_2O	ad		1000 ml
pH 7.5			

2.5.3 DNA-DNA Hybridization

In order to neutralize unspecific binding sites (i.e. reduce background), the membrane was pre-hybridised with 20 ml of freshly prepared pre-hybridization solution for 2 h at 42°C in an hybridization oven. The membrane was incubated in a hybridization oven overnight at 42°C with hybridization solution and 15 µl of the probe (labelled *mer operon*). Before the probe was added, it was heated for 10 min at 95°C and put on ice in order to melt the DNA and generate single strands.

Pre-hybridization Solution

50% formamide/ 0.1% Na-laurylsarcosine	16	ml
Blocking Reagent	4	ml
10% SDS	80	µl

Hybridization Solution

10% formamide	50	ml
20xSSC	25	ml
10% Na-laurylsarcosine	1	ml
dH_2O	ad 100	ml

For hybridization 4 ml of Blocking Reagent and 80 µl of 10% SDS were added to 16 ml of Hybridization Solution.

Blocking Reagent

Buffer1 (see below)	200	ml
Blocking Reagent	20	g

Buffer1

Maleic Acid	0.1	mM
NaCl	0.15	mM
dH_2O	ad 1000	ml
pH	7.5	

The Blocking Reagent (Boehringer Mannheim) was autoclaved immediately after preparation and stored at 4°C.

2.5.4 Probe Detection

For the detection of the probe, the hybridised membrane was washed twice for 5 min at room temperature with 2xSSC/ 0.1% SDS and twice for 15 min at 70°C with 0.1xSSC/0.1% SDS. It was then immersed in Wash Buffer at room temperature for 5 min and 30 min at room temperature in 80 ml Buffer2. The solution was discarded and the membrane incubated at room temperature in 20 ml of Antibody Solution (20 ml Buffer2 and 3 µl Anti-digoxigenin-AP-Fab fragments). Then, the membrane was washed twice for 15 min in Wash Buffer and once for 5 min in Buffer3. At last, the membrane was incubated for 10 min in 10 ml CSPD-Solution (10 µl CSPD on 10 ml Buffer3). CSPD is dephosphorylized by Alkaline Phosphatase which renders a phenolate anion that disintegrates and emits light in the course. The membrane was carefully dried with a paper towel, sealed in plastic foil (autoclave bag #759705, Brand GmbH & Co, Wertheim, Germany), laid on film (Kodak Omat x-ray). Different films were developed after 30 min, 2 and 4 h.

Wash Buffer

Buffer1	1000	ml
Tween-20	3	ml

Buffer2

Buffer1	85	ml
Blocking Reagent	15	ml

Buffer3

Tris/HCl	15.78	g	0.1	M
NaCl	5.84	g	0.1	M
dH_2O	ad 1000 ml			
pH 9.5				

20xSSC

NaCl	175.32	g	3	M
Na-Citrate	88.23	g	0.3	M
dH_2O	ad 1000 ml			
pH 7.0				

2.6 PCR-Temperature Gradient Gel Electrophoresis (TGGE)

DNA fragments of identical length can be separated by temperature gradient gel electrophoresis (TGGE; Muyzer and Smalla, 1998) according to their melting behaviour that is dependent on their base composition. The DNA molecule melts in discrete segments also known as melting domains. Differences as little as a single base can be detected with TGGE in all but the final domain (which holds the DNA together, preventing dissociation into single strands). Thus, a supplementary high melting domain can be added to one end of the PCR product by addition of around 40 G and C residues to one of the primers. This so called GC clamp is usually added to the 5' end, as degeneracy at the 3' end of a primer is prone not to be tolerated and extension from the primer hindered (see Table 2-4 for the primers used).

2.6.1 PCR for TGGE Analysis of Total Bacterial Community (16S rDNA)

PCR for TGGE was carried out with AmpliTaq DNA Polymerase Stoffel Fragment (Perkin Elmer), a recombinant polymerase with no associated 3' to 5' nuclease activity and enhanced thermal stability. The 20 µl reaction mixture contained 1× Stoffel Buffer (Perkin Elmer-Applied Biosystems, Foster City, USA), 3 mM $MgCl_2$, 0.05 volume Dimethylsulfoxid

(DMSO, p.a.), 0.2 mM dNTPs, 100 nM of each primer (U968-GC1 & R-1401, Table 2-4), 5 units of Stoffel Fragment and between 0.5 to 2 µl of community DNA template. The 16S rDNA was amplified using a 7 min denaturation step at 94°C, 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C. The final extension step was performed for 10 min at 72°C. One µl of PCR products was visualized in an 0.8% agarose gel.

2.6.2 PCR for TGGE Analysis of Mercury-Resistant Community (*mer*A)

Amplification was performed with the Eppendorf Mastercycler Personal (Hamburg, Germany) using an initial touchdown PCR step of 10 cycles of 94°C for 10 s, 59 - 55°C (decremental with a decrease of 0.5°C per cycle) for 20 s and 68°C for 20 s, afterwards continuing with 45 cycles of 94°C for 10 s, 46°C for 20 s and 68°C for 20 s. The 20 µl PCR reactions contained 10 mM Tris-HCl (pH 8.3), 5% DMSO, 50 mM KCl, 3 mM MgCl2, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.4 µM of forward and reverse primer (GC-merAfor & merArev, Table 2-4) respectively, 0.5 units of recombinant Taq DNA polymerase (Qiagen, Hilden, Germany), and 1 µl of DNA template as purified from the microcosm samples (see 2.8.5).

2.6.3 Culture-Independent Detection of *Ps. putida* KT2442::mer73 by Quantitative, Competitive PCR (cPCR)

Quantification of the GEM using PCR offered an alternative to the cultivation-dependent determination of cfu. It renders quantitative information about the availability of DNA carrying the mercury resistance. For competitive PCR, a standard resembling, however, not identical to the sample template was added to the reaction mixture and co-amplified. In contrast to the sample template, the standard was of known concentration. The products were separated after PCR and the template concentration could be determined in accordance with the signal of the standard. In this particular approach, the DNA sequence of the standard resembled the target nearly completely except for a thymine that was replaced by a guanine, drastically altering the melting behaviour of the amplicon (Felske *et al.* 2001) and thus enabling separation of the two DNA sequences by TGGE (see 2.5). Standard DNA was generated by PCR with primers (Table 2-4) that incorporated the base replacement and with the genomic DNA of *Ps. putida* KT2442::mer73 serving as template. The quantitative PCR reaction as well as TGGE procedures were carried out as described in Felske *et al.* 2001. To five identical PCR mixtures containing water or sediment samples from the GEM inoculated microcosms different amounts of standard were added [40 fg, 40/3 fg, 40/9 fg, 40/27 fg, and 40/81 fg] and PCR performed with the primers PROb & PROp (Table 2-4). After the reaction,

amplification products were separated with TGGE (see below) and GEM concentration determined by comparison with the standard signals.

2.6.4 TGGE Protocol

For separation 5 µl of the PCR products mixed with 1 µl of loading dye were loaded on a 0.8 mm polyacrylamide gel that was prepared one day prior to the TGGE with 1× MOPS buffer (20 mM 3-morpholinopropanesulfonic acid, 1 mM EDTA, pH 8.0) and exposed to a 37°C to 47°C (sediment samples) or 35°C to 45°C (water samples) thermal gradient with a fixed voltage of 350 mV (ca. 28 mA) for 6 h in a Diagen TGGE system (Qiagen, Hilden, Germany). The gel was prepared according to the following protocol. For PCR products from microcosm sediment and water samples gel and electrophoresis was carried out with MOPS Buffer, for *mer*A amplicons MN Buffer was used.

Acrylamide Gel

dH2O	9 ml	
Urea	21.6 g	(8 M)
Glycerol 87%	1 ml	(2% v/v)
50x MOPS Buffer/50×MN Buffer	0.9 ml	
Acrylamide Stock Solution	9 ml	(6% w/v)
37% Formamide	9 ml	(20% v/v)
TEMED (N,N,N',N'-Tetramethylethylendiamine)	77 µl	
10%(w/v) APS (ammonium persulfate)	135 µl	

50× MOPS Buffer

MOPS (3-morpholino-propane-sulfonic acid)	1 M	
EDTA	50 mM	
pH 8.0		

1× MN Buffer

MOPS	20 mM	
NaOH	10 mM	

Loading buffer for TGGE

MOPS	400	mM
Urea	8	M
EDTA	10	mM
Bromphenolblue	0.08%	
Xylencyanol	0.08%	

The *mer*A PCR products (5 µl), usually approximately 280 bp in length, were separated in principle as described above against a temperature gradient of 36 to 51°C. A pre-run of 10 min at 10 V was performed to allow gradient stabilization, and was followed by a 3.5 h electrophoresis at 400 V.

After competitive PCR, 2 µl of the reactions were loaded on a polyacrylamide gel as described above and submitted to a fixed current of 25 mA (approx. 480 V) and a temperature gradient of 36°C to 52°C for 2 h. The TGGE gel was a 0.8 mm-polyacrylamide gel as described above but with 9% acrylamide and with 1× MN buffer.

2.6.5 Silver Staining

After the run, the gel was fixed for 3 min in 10% ethanol/ 0.5% acetic acid and then stained with 0.2% $AgNO_3$ (dissolved in 10% ethanol/ 0.5% acetic acid). The gel was developed with 3% NaOH containing 0.005 volume formaldehyde. Gels were conserved with a solution containing 10% glycerol and 25% ethanol, including an appropriate sheet of cellophane. The gel covered by the cellophane sheet was dried overnight in a geldryer plastic frame at room temperature or 50°C.

2.6.6 TGGE Standard

The TGGE standard (Heuer *et al*. 1997) was composed of 16S rDNA PCR products from *Erwinia carotovora* subspec. c*arotovaora* (A), *Agrobacterium tumefaciens* (B), *Erwinia herbicola* (C), *Burkholderia gladioli* (D), *Streptomyces aureofaciens* (E), *Actinomyces* sp. strain QMB-814 (F), *Clostridium pasteurianum* (G), *Rhizobium leguminosum* (H), *Actinosynnema mirum* (I), *Actinoplanes auranticolor* (J), and *Pseudomonas fluorescens* R2f (K; Figure 2-1).

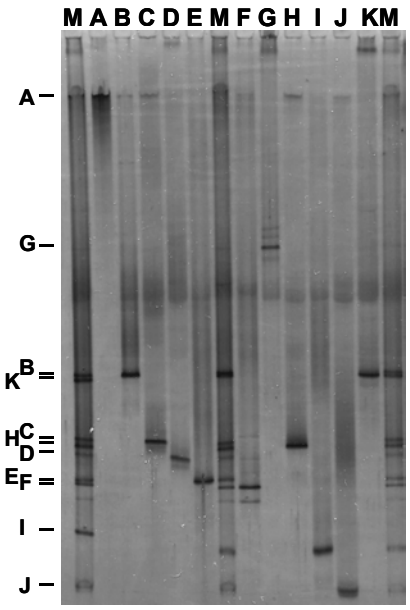


Figure 2-1 Composition of 16S rDNA standard used in Thermo Gradient Gels. See text for the strains matching the 16S rDNA bands in lanes A - K. A combination of the marker strains was run in the lanes designated M. The most protruding bands are associated with the organisms from which they derive, however, most organisms can be matched with several bands of which not all are indicated for lane M.

2.7 Sequencing

DNA sequencing was carried out with ABI PRISM™ 377 und ABI PRISM™ 3100 Genetic Analyser exploiting the dideoxy mediated chain termination method devised by Sanger *et al*. (1977). Dideoxynucleotide sequencing utilizes 2',3'-dideoxynucleotide triphosphates (ddNTPs) that differ from deoxynucleotides by having a hydrogen atom attached to the 3' carbon instead of an OH group. Thus, they are unable to form a phosphodiester bond with the next deoxynucleotide. For the detection of the termination fragments, fluorescein-marked terminators were employed which consist of a donor that takes up energy emitted from an argon laser of the sequencing unit and transfers it without loss to an acceptor within the same molecule. The acceptor emits light of a wavelength that is specific for the termination fragment and is recorded by a photosensor. The sequencing reaction was carried out in a total volume of 20 µl with approximately 75-100 ng of the purified (QiaEx or PCR purification kit, Qiagen, Hilden Germany) DNA template, 10 pmol of the sequencing primer, 6 µl of "Big Dye Terminator™ v. 2.0 Ready Reaction Mix" (containing the terminators, dideoxynucleotide triphosphates, $MgCl_2$, and AmpliTaq® DNA Polymerase FS). Termination fragments were produced in 25 cycles of PCR in an Eppendorf Mastercycler®Personal (Hamburg, Germany)

with 15 sec for strand denaturation at 96°C, 15 sec for primer annealing at 55°C and 240 sec of primer extension at 60°C. The termination fragments were ultimately precipitated by addition of 1/10th volume of 3M Na-acetate (pH 5.3) and 2 volumes of 98% (v/v) ethanol, thorough mixing and centrifugation at maximum speed (20000×g). The supernatant was withdrawn, and the resulting pellet washed with 70% (v/v) ethanol. Eventually, the DNA pellet was vacuum dried (DNA Speed Vac-DNA120 SAVANT SS1, Savant Instruments, Farmingdale NY, USA) and stored at -20°C until sequencing. For the separation of the termination fragments, the DNA pellet was resuspended in 20 µl of Hi-Di Formamide (Perkin Elmer Biosystems, Foster City, California, USA) and loaded for electrophoresis on the sequencing gel (4.25% polyacrylamide, 48 cm).

2.7.1.1 Data Recording and Sequence Analysis

Fluorescent signals were automatically converted to sequence data with the software package purchased with the ABI PRISM 337 DNA-Sequencer or the ABI PRISM Genetic Analyser (ABI PRISM™ 377 Data collection Version 2.1 and Sequence Analysis, Version 3.2, or ABI PRISM™ 3100 Data collection Version 1.0.1 and Sequence Analysis, Version 3.7, Perkin Elmer Applied Biosystems, Weiterstadt, Germany). Sequence evaluation and first corrections were done with Chromas Version 1.41 (Brisbane, Australia) by comparing the automatically extracted sequence and chromatograph signals.

2.8 Microcosm Experiments

2.8.1 Microcosm Design

Stream microcosm design was adapted from Vogel and LaBarbera's design of flow tanks (1978) and Beyers and Odum (1993). The microcosms (Figure 3-1) consisted of acrylglas troughs (700 mm × 150 mm × 250 mm) with a loose lid. Upstream and downstream collimators were placed 75 mm from each end and were made of 20 mm thick Plexiglas plates with 2000 holes (Ø 3mm) drilled into each plate. Inflow and outflow of water were directed through funnels at each head side of the microcosm. The funnels possessed an opening with a diameter of 15 mm on one end, and opened into the microcosm with a diameter of 75 mm over a length of 100 mm. For the recycling and water inflow PharMed 6485 tubing was used with an inner diameter of 7.9 mm and 3 mm walls. Water was pumped by an L/S®(Laboratory Standard) Masterflex Console Drive with a capacity of 6 - 600 revolutions

per min. The drive was provided with an eight channel pump head that contained four cassettes for tubing. Water was recycled through the system at 1000 ml min^{-1}.

Drainage of superfluous water was regulated by water level. The water was drained through three 15 mm holes at the downstream head of the microcosm.

Inoculation with bacteria was accomplished by inserting silicon tubing from the chemostat with a long needle (Braun, Melsungen, Germany, Sterican, 0.90 × 70 mm, 20 G × 2 4/5" Luer Lock) into the microcosm through the middle of the three 15 mm diameter rubber stoppered holes at the upstream head side.

Pressurised air was pumped into the pre-chamber through a 25 mm × 25 mm × 25 mm aquarium aeration stone that was connected with an aquarium pump (WISA 300, Germany; 300 L h^{-1}) through silicon tubing (Figure 3-2). The aeration stone also helped distributing the inflowing water over the area of the collimator.

The microcosms were filled with ca. 12.5 L of Elbe River water that was continuously replaced with tap water during a run at a refreshment rate of 15 ml min^{-1} (which translates into a complete exchange of water within 14h). Non-chlorinated drinking water from the tap was low calcium water derived from a dam with an associated reservoir in the Harz mountains in Germany. The main chamber was provided with 4 cm of Elbe River sediment (i.e. 3 L, bearing 3.4 kg wet weight or 1.2 kg dry weight). With these settings (9 L over 50 cm at 1.03 L min^{-1}) current velocity was calculated to be 5.6 cm min^{-1} (or 9.3×10^{-4} m sec^{-1}).

The microcosms were kept at room temperature, water temperature was constant at 22°C, pH was 7 as determined with pH-indicator strips (Merck, Darmstadt, Germany; pH 0-14). To control primary production, the side walls of the troughs were darkened with paper cartons, and a tubular fluorescent plant grow light (Lampi-1915-1, 50 cm, 15W) was employed parallel to and approximately 1.0 m above the microcosms to render a 12:12 h light:dark cycle.

2.8.2 Flow Visualization

In rivers laminar flow of water is very uncommon. Generally, flow is turbulent, except at channel boundaries where the current is very low (Allan, 1999). However, if flow in the microcosms was too turbulent, abrasion would prevent settling of bacteria. Therefore, the microcosm was tested for its flow behaviour to ascertain that flow was relatively even and neither still regions of no flow nor regions of extreme turbulence existed. Through the microcosm that was filled with sediment water was recycled at 1 L min^{-1}. One Pasteur pipette full (2 ml) of a saturated crystal violet solution (1% w/v) was added directly into the inflow of

water in the pre-chamber (Figure 3-2, first picture at time 00 sec). The distribution of the dye was documented by taking serial images with a digital camera (Nikon COOLPIX 950).

2.8.3 Inoculation

Two of the microcosms were inoculated from continuous cultures of *Ps. putida* KT2440 or *Ps. putida* KT2442::mer73, respectively. The chemostats consisting of 1 L Schott bottles with an olive at the bottom of the flask for withdrawal of culture suspension and another olive above the 1 L mark for implementation of a sterile filter were fed with M9 minimal medium (Wagner-Döbler *et al.* 1992) that was amended with 10 mM sodium benzoate at 30 ml h^{-1}. A stirrer provided for good mixing and aeration of the culture. Medium was added via a sterile steel needle through a silicon seal matching the Schott PBT (Polybutylene Terephtalate) lids. Cell density in the chemostats was approximately 10^7 cells ml^{-1}. The sterile filter allowed for sterile air to be respired. Bubble traps were used to prevent contamination of the medium from the chemostat and contamination of the chemostat from the microcosm.

2.8.4 Elbe River Sampling and Recycling Period

For the first of two microcosm experiments water and sediment were collected in July 2000 from the Elbe River near Magdeburg, Germany, at the km 320 measuring station. Water temperature was 17.9 °C, water pH 6.7, and oxygen contents 12.9 mg L^{-1}. Surface water velocity was determined at the measuring station approximately 10 m from the river bank by dropping Styrofoam crumbs and measuring the time they needed to travel 10 m down the river. The surface water flow velocity was 0.45 m sec^{-1}. The acid-soluble Hg-concentration in water was 0.06 ppb, the Hg-concentration of SPM-borne sediment was approximately 7 mg kg^{-1} (http://www.arge-elbe.de). The sediment was scraped off the bottom of the Elbe River at the same site that the water was removed from, about 3 - 5 m into the river off the shore, where water depth was about 1.5 m. The sediment appeared smooth and of dark brown colour (silt). Black sediment was avoided as it was assumed to be mainly anaerobic. The sediment was sieved through a 5 mm sieve before addition to the microcosm.

For the second microcosm experiment water and sediment were collected in October 2000 from the Elbe River near Magdeburg at the same site. Water temperature was 11.6 °C, water pH 8.3, and oxygen contents 10.7 mg L^{-1}. The sediment was sieved through a 5 mm sieve before addition to the microcosm.

The water was recycled for ten days during the first microcosm experiment and for twelve days in the second microcosm experiment without addition of fresh water or inoculants so that

the disturbed bacterial community could re-establish. Samples were taken regularly during this period.

2.8.5 Sampling and Sample Treatment

Water samples of 10 ml were taken with a glass pipette and transferred into a 50 ml tube (Greiner, Frickenhausen, Germany). After the addition of 1 ml sodium-acetate (3 M, pH 5.8) and 20 ml absolute ethanol to destroy bacterial cell membranes they were stored at –20°C.
Sediment samples were taken with a sterile 5 ml plastic pipette equipped with a blunt end. The pipette was inserted into the sediment at the centre of the microcosm and 5 ml of sediment were drawn in and transferred into a 50 ml tube (Greiner, Frickenhausen, Germany). The sediment was weighed (wet), mixed with Phosphate Buffer (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 ad 1 L, pH 7.3), in a 1:1 (w(g):v(ml)) ratio, vortexed for min, sonicated in a water bath (Sonorex Super RK510H, Bandelin electronic, Berlin, Germany) for 2 min and again vortexed for 1 min. When the sediment had settled (after 15 - 30 min), 1.5 ml of the supernatant were transferred into a reaction tube and a dilution series prepared with 1% NaCl for determination of colony forming units (cfu).

2.8.6 Determination of Colony Forming Units (CFU)

Aliquots (100 µl) from three serial dilutions (in 1% NaCl) of water or sediment samples from the microcosms were spread in triplicate on solid R2A (Fluka Chemie, Buchs, Switzerland) agar medium for the determination of total cfu. For the determination of mercury-resistant bacterial numbers, samples were spread as described above on solid R2A agar containing 5 ppm Hg ($HgCl_2$). GEM numbers were followed by spreading three serial dilutions of the samples in triplicate on solid M9 medium containing 10 mM sodium benzoate (Fluka, Buchs, Switzerland) as sole carbon source, 100 µg ml^{-1} Rifampicin and 1 ppm Hg ($HgCl_2$). Spreading of water or sediment samples not containing the GEM did not result in colonies on the selective plates. The detection limit was determined at 10 cfu ml^{-1}.

2.8.7 Statistical Analysis of Cell Densities

Each data point (Figure 3-7 & Figure 3-8) represents the average from triplicate plating. Although samples were spread in three serial dilutions, great care was taken that the data shown for the different sampling dates were always representing the same dilution (10^{-3}) to minimize sources of error and to maximize identical treatment of the samples shown in the figure. Nevertheless, cfu determined in differently diluted samples resembled each other, usually not deviating more than by a factor 2 to 3. The standard deviation was determined

with Equation 1 for each data point, where n is the number of data points contributing to the average (3 for triplicate plating) and x_i is the cfu value for one out of three platings.

$$s_{n-1} = \sqrt{\frac{n\sum_{i=1}^{n} x_i^{\,2} - (\sum_{i=1}^{n} x_i)^2}{n(n-1)}}$$

Equation 1

To test if the sum of the difference between two curves differed statistically significantly from zero, i.e. if the curves from the three microcosms were essentially the same, the t-test for paired observations was performed (Equation 2, compare Sachs 1968). This test can be applied if the samples are dependent, e.g. when they originate from the same sample but are treated differently in the course. This premise was satisfied in the experiment. Since three curves had to be compared, this test was repeated for all three combinations (microcosm 1 *vs.* microcosm 2; microcosm 1 *vs.* microcosm 3; microcosm 2 *vs.* microcosm 3, Table 3-1). The t-test is quite robust so that minor deviations from normality will have minor effect on the power of the test. The Null hypotheses were stated as the difference of the mean cfu of one microcosm (e.g. $\bar{x}$) and that of another microcosm (e.g. $\bar{y}$) being equal to zero: $H_{0\text{-}1}$: $\bar{x} - \bar{y} = 0$; $H_{0\text{-}2}$: $\bar{x} - \bar{z} = 0$; $H_{0\text{-}3}$: $\bar{y} - \bar{z} = 0$ and all three t-values compared against the critical t-value for a two-sided t-test at a significance level of α=0.05 (Table 3-1) and 15 (total bacterial densities in water) or 16 (all other curves) degrees of freedom. The degrees of freedom describe the number of differences that may be estimated with the statistical parameters known. In this case the degrees of freedom are calculated with df=n-1. Since n differed for the determination of total bacterial densities in water, the df differed also.

The t-value was calculated according to Equation 2, where n is the number of data pairs and $\bar{x}_i$ / $\bar{y}_i$ are the average data points for two specific microcosms at a specific sampling day i.

$$t = \frac{\sum_{i=1}^{n} (\bar{x}_i - \bar{y}_i)/n}{\sqrt{\frac{\sum_{i=1}^{n} (\bar{x}_i - \bar{y}_i)^2 - (\sum_{i=1}^{n} \bar{x}_i - \bar{y}_i)^2 / n}{n(n-1)}}}$$

Equation 2

2.9 Fluorescence Detection

Fluorescent cells were observed by microscopy or fluorescence determined in a spectrophotometer.

2.9.1 Fluorescence Microscopy

Ps. putida KT2440::*mergfp c*onstruct colonies, that were able to grow with mercury on M9 minimal agar medium (+benzoate), were examined with a Zeiss-Axioplan Fluorescence Microscope (Oberkochen, Germany) with a FITC Filter Set 487910 (excitation 450-490, emission 515-565) and 2.5× magnification (Plan-Neofluar objective). Source of excitation was a 50 W (A/C) high pressure mercury lamp (Osram HBO50). The microscope was connected with a digital camera (Intas, Göttingen, Germany) that recorded the fluorescent colonies and a live picture was displayed on the monitor with the software Intas Camera Control 1.32a. The camera adjustments were set on contour, 1/1 shutter mode, and the gain was 12 dB. The microscopic picture was shown in RGB colours. Liquid cultures could be recorded likewise, however, bacterial cells were then observed with 40× or 100× magnification (Plan-Neofluar objective).

2.9.2 Fluorescence Spectrometry

In order to assess fluorescence of the new *Ps. putida* KT2440::*mergfp* constructs in liquid culture, 1 ml of cells grown in M9 minimal medium (+10 mM benzoate) was transferred to a quartz cuvette and assessed in a fluorescence spectrophotometer (RF-5000, Shimadzu Corporation, Spectrophotometric Instruments Division, Kyoto, Japan) with excitation at 475 nm and detection at 515 nm. Both, M9 minimal medium (+10 mM benzoate) and dH_2O were repeatedly measured to determine a blank and the measure was subtracted from the measurement of the sample.

2.10 Kinetics of Mercury Reduction

The capability of the constructs to reduce mercury chloride was determined in a kinetic online measurement assay using cold vapor atomic absorption spectrometry (CVAAS; AAS 2100 Perkin Elmer, Überlingen, Germany). Detection was at 253.7 nm, the gap opening was 2 nm and a 5 mA Hollow Cathode Lamp (HLC) was the source of light. The detection limit was 0.4 $\mu g\ L^{-1}$. Measurements were compared with a calibration curve obtained by chemical reduction of a standard mercury solution (1 $g\ L^{-1}$ $HgNO_3$) with $SnCl_2$. Mercury transformation

rates increased in a linear fashion between 0 and 14 mg L^{-1} Hg. The data were recorded with a dot matrix printer (Perkin Elmer EX800).

For kinetic studies, precultures of the constructs were grown overnight in M9 (+benzoate) medium without mercury, inoculated 10% into fresh M9 (+benzoate) medium without mercury and grown to an optical density of 1.3 at 600 nm (late exponential phase). Mercury reduction of $Hg^{2+}Cl_2$ to Hg^0 was initiated by injecting 1 ml of cell suspension into 5 ml M9 (+benzoate) medium amended with mercury (3 or 6 mg L^{-1} Hg^{2+} as $HgCl_2$). The measurements were carried our in triplicates, each vessel (Nalgene, polycarbonate, 50 ml) was incubated at 30 °C for 2.5 min after injection. The cell suspension was mixed inside the vessel with a magnetic stirrer at 350 rpm and ultimately aerated vigorously with compressed air for 0.5 min to blow the produced Hg^0 into the CVAAS. This procedure was repeated 4 times for each vessel for a full measurement.

Before and after the injection, the cells were spread on LB agar medium in order to determine the number of cultivatable cells injected into the mercury solution and to elucidate the fraction of cells surviving the treatment.

2.11 Statistically Correlating Mercury Reduction and Fluorescence

The correlation coefficient was calculated for mercury reduction and fluorescence with the following equation to investigate a possible link between mercury reduction and fluorescence.

$$r_{x,y,} = \frac{\mathrm{cov}(x,y)}{s_x \bullet s_y}, \text{ with } -1 \le r_{x,y} \ge 1 \text{ and } \mathrm{cov}(x,y) = \tfrac{1}{n}\sum_{i-1}^{n}(x_i - \bar{x}) \bullet (y_i - \bar{y})$$

Equation 3

The suffixes x and y stand for the parameters that are correlated, s is the standard deviation and $\bar{x}$ is the average of the items within parameter x or y, the values of an individual item are x_i, y_i.

3 Results

3.1 Stream Microcosm Experiments

Knowledge of the genetic modification in a GEM is important as it might explain deviating behaviour of the GEM from the wildtype or parent. The deduced effects of the modification remain, however, mere speculation, unless the behaviour of both, modified and unmodified strain, are examined under specified conditions. Here, the influence of the GEM on the indigenous bacterial community in river water and sediment microcosms was investigated to evaluate its safety in the situation of a release.

Two main microcosm experiments were conducted. The first experiment was carried out to examine the survival of the GEM and its effect on the indigenous microbial community. The second experiment was performed to investigate gene transfer in the presence or absence of phenyl mercuric acetate (PMA) as selective pressure. In the following these experiments will sometimes be referred to as first and second microcosm experiment.

3.1.1 Stream Microcosm Design

A beaker filled with water and a hand full of sediment (and sometimes leaves) can be sufficient to make a microcosm (e.g. Bale *et al.* 1988, Iwasaki *et al.* 1993, Muela *et al.* 1994, Sobecky *et al.* 1996, Leff *et al.* 1997). These so called 'simple' microcosms have the benefit that experiments carried out in them are relatively easily controlled, since comparably few parameters influence the course of events during the experiment. In terms of simplicity, reproducibility, and costs, such a microcosm would be the system of choice. However, it encompasses only few features of the environment under study and largely ignores most other parameters. 'Complex' microcosms on the other hand attempt to include more of the physical and chemical variables and dynamics associated with the environmental habitat. This complexity irrevocably results in quite extensive experimental set-ups (e.g. Rochelle *et al.* 1989, Wagner-Döbler *et al*. 1992, Hill *et al* 1994, Ashelford *et al*. 1995, Pauling & Wagner-Döbler, submitted) paying tribute to the multilayered nature of the habitat. For this work stream microcosms were best capable to represent the characteristics of a river in the laboratory (Warren & Davis 1971).

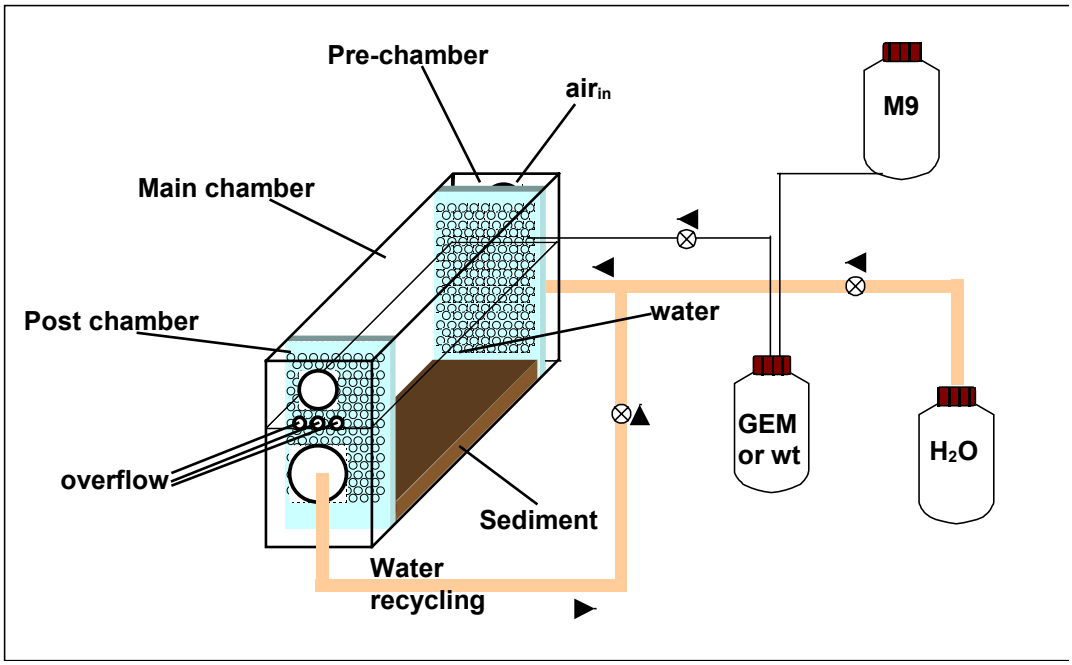


Figure 3-1 Schematic view of the stream microcosm. The GEM or the parent strain *Ps. putida* KT2440 ("wildtype" = wt) or both were cultivated respectively in chemostats containing M9 minimal medium (Wagner-Döbler *et al*. 1992) with benzoate as sole carbon source. The microorganisms were added continuously to the respective microcosms. Water and inoculated bacteria were mixed in the pre-chamber by bubbling air in through an aeration stone.

For the stream microcosm constructed for this work, downstream flow was achieved by the continuous addition of water. To increase water flow and to avoid build-up of bacterial density- or nutrient- gradients, the water was recirculated. A plant light was implemented to control primary production and water temperature was constant at 22°C. The microcosms were filled with near riverbank sediment and water from the Elbe River at Magdeburg. After a recycling period of 1½ weeks during which a stable bacterial community could establish, the microcosms were continously fed with tap water and superfluous water was drained. Inoculation occurred continuously from chemostats. For more technical information refer to chapter 2.8.1.

Many stream microcosms in the literature have been constructed to be used outdoor (e.g. Schulz & Liess 2001, Dodds *et al*. 1999). The microcosms described here (Figure 3-1) were built to be operated indoor, in a laboratory. Three parallel microcosms (each 70 × 15 × 25 cm in length, depth and height) could easily be fit under a fume hood (ca. 150 × 70 cm in length and depth), allowing research involving toxic and volatile compounds (PMA was used in the second microcosm experiment).

3.1.2 Flow Characterisation of the Stream Microcosm

The rationale of constructing a new stream microcosm was to develop a system that would well represent near-bed conditions of a river to carry out experiments regarding the impact of *Ps. putida* KT2442::mer73. Requirements were that the flow of water through the microcosm be relatively laminar to allow bacteria to settle to the sediment and avoid abrasion by turbulent flow. Flow velocity was to resemble speeds that can be found in nature, although flow velocities close to the sediment are regularly slow.

Therefore, examination of the microcosm concerning its flow characteristics was mandatory before carrying out the impact experiments. Flow visualization experiments were carried out by pipetting a saturated solution of Crystal Violet into the inflow (see 2.8.2).

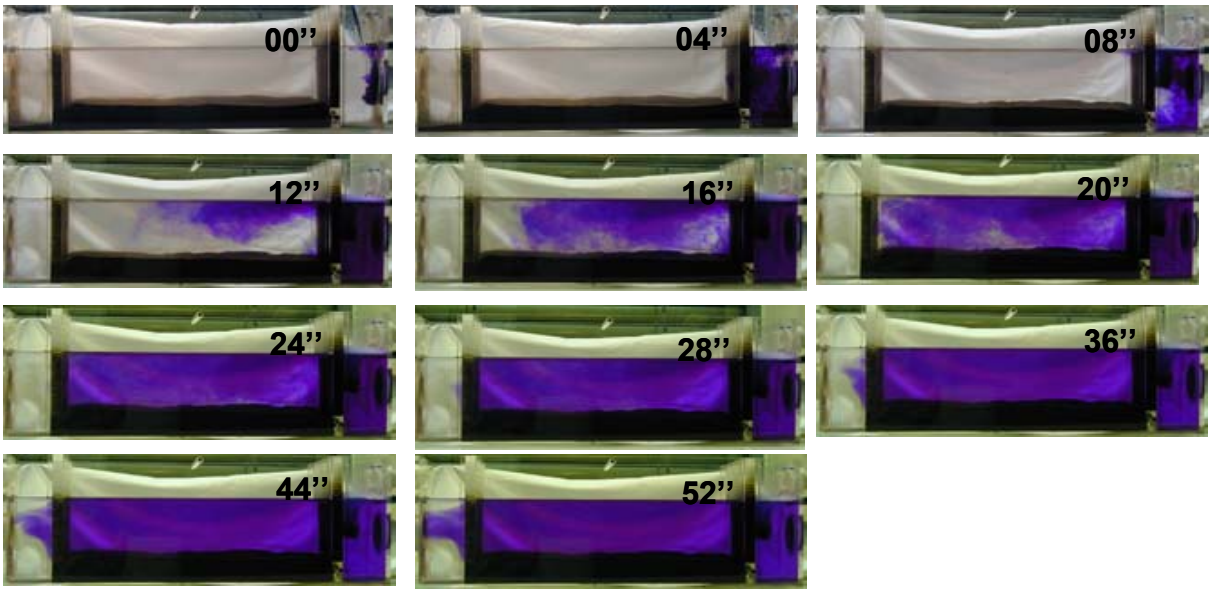


Figure 3-2 Visualization of Flow in the Stream Microcosm. A saturated crystal violet solution was added to the pre-chamber via a Pasteur pipette and the time course of its distribution photographed. Time-lapses between the pictures were as indicated. Good mixing of dye and water could be observed before the crystal violet entered the main-chamber. Surface flow was visibly quicker initially, however, a dye front reaching down to the sediment bottom reached the outflow collimator. Further mixing of dye and water in the main-chamber accounted for attenuation of outflow. Turbulence of water flow was transitional with a Reynolds number between 400 and 1650.

During the addition of crystal violet to the microcosm, it could be observed that flow within the funnel (connecting the tube with the acrylglas trough) was spiralling (data not shown). The aeration stone supplying air to the system was placed directly in front of the inflow funnel and helped to distribute the water within the pre-chamber before it would flow through the collimator (Figure 3-1). Air bubbles from the aeration stone affected the current adding an

upward momentum. Thus flow appeared first at the surface in the main chamber and a clear spot remained in the lower part of the water column for about one third of the main chamber length. This spot successively filled with dye coming from the pre-chamber as well as through some backward flow as current progressed.

Since turbulence is a major parameter in rivers controlling such events like settling of bacteria and availability of nutrients, the Reynolds number is an important measure. The Reynolds number which describes the turbulence of a moving gas or liquid is given in the following equation:

$$Re = \frac{UL}{\nu}$$

Equation 4

where U is the velocity of the water (here 9.3×10^{-4} m sec^{-1}), L is the characteristic length, i.e. the length of the main chamber in our case (0.5 m), and ν is the kinematic viscosity (1.004×10^{-6} m^2 s^{-1} for freshwater at 20°C). With these parameters the Reynolds number was 463, indicating laminar flow. Only at Reynolds numbers greater than 2000 flow is turbulent (Vogel 1989). However, Allan (1995) termed "transitional flow" in addition to laminar and turbulent flow if the Reynolds numbers lies between 500 and 10^3-10^4. Visually determined velocity in the main chamber was considerably higher than theoretically expected (Figure 2). Passage of the dye through the main chamber took approximately 24 sec, i.e. flow speed was 3.3×10^{-3} m sec^{-1}, which was nearly a factor 4 quicker. With this velocity the Reynolds number computed to 1650, for which flow could be considered transitional. This discrepancy between calculated and visually determined flow velocity can be attributed to differences in local flow patterns. For example a nearly still area could be observed in the bottom part of the post-chamber reducing the effective volume in the velocity calculations. A delayed entrance into the main chamber or the post-chamber could be seen at the collimators. For these reasons, water velocity in the main chamber was considerably higher than theoretically calculated for the whole microcosm system.

3.1.3 Preliminary Microcosm Experiments

Before starting with the microcosm experiments, several questions needed clarification. Was there a bacterial density gradient over the length of the microcosm? In this case sampling at different positions in the microcosm would have been valuable. What would be the most effective method to remove bacteria that were attached to sediment particles? This problem

has already been discussed in 1.3.1. In the second microcosm experiment one microcosm was operated with PMA to create a selective pressure. What concentration should be used? The results of experiments that were performed to give answer to these questions are presented in the following.

3.1.3.1 Bacterial Density Profile over Microcosm Length

Differences in cell densities at various positions of the microcosm were investigated. Sediment samples were taken from all three microcosms along the length of the microcosm, at the start (ca. 5 cm behind the first collimator), in the middle and towards the end (ca. 5 cm before the second collimator), and total bacterial densities as colony forming units (cfu) were determined (Figure 3-3).

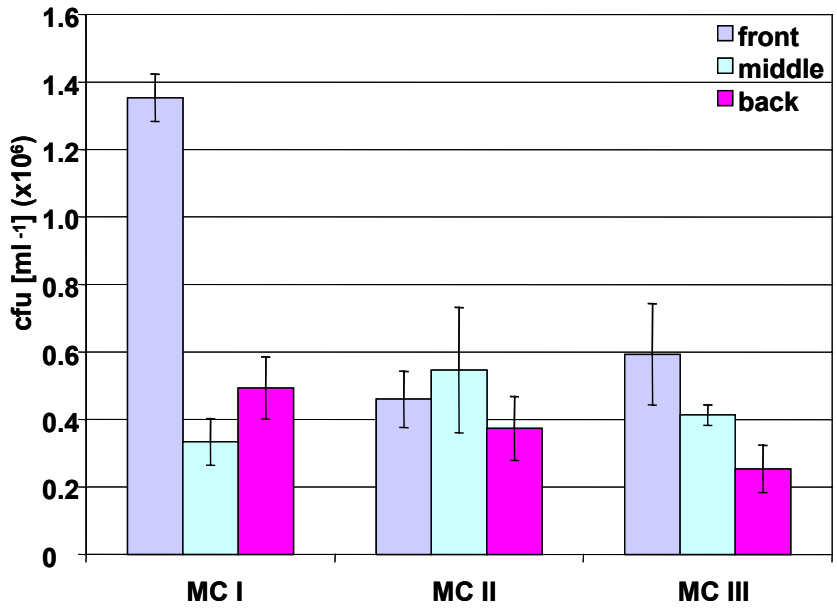


Figure 3-3 Total Bacterial Densities over the Length of the Microcosm. Bacterial densities are presented as determined for different positions within the three microcosms. See text for further information.

Densities were similar within a factor five for all nine sampling spots, and eight out of nine were between 2×10^5 and 6×10^5. At the beginning of MCI, bacterial densities were appreciably higher. Nevertheless, higher bacterial densities in comparison to the other two positions in all three microcosms could not be found for any of the sampling positions. Since no favoured position for bacterial growth could be discriminated within the microcosms, both, in the first and the second microcosm experiment, sediment samples for the determination of bacterial densities were taken from the middle of the microcosms.

3.1.3.2 Detachment of Bacteria from Sediment Particles

For the determination of total or mercury-resistant colony numbers in sediment it was essential to detach the bacteria from the sediment particles and transfer them to a liquid phase that could be diluted and easily spread on agar medium. Cells can be removed from soil or sediment particles by vigorous shaking (on a vortex at full speed, 2500 rpm, MS 1 Minishaker-Vortexer, IKA-Werke GmbH & Co. KG, Staufen, Germany) or by sonication (Sonorex Super RK510H Bandelin, Berlin, Germany). Wagner-Döbler *et al.* (1992) extracted bacteria from sediment by diluting the sediment sample 1:4 (w/v) in phosphate buffer and shaking it on a vortex for 1 minute. To test what treatment would most successfully extract the cells from the sediment, sediment samples were diluted 1:1 in PBS Buffer and subjected to sonication or vigorous shaking for different periods of time (Figure 3-4). The treatment was carried out in duplicate and each treatment sample spread in three serial dilutions and triplicates as described in 2.8.6. Two minutes of sonication or vigorous shaking yielded the greatest number of cfu. While extension of vigorous shaking did not seem to have much of an effect, two min of sonication rendered nearly 10 times as many colonies as 1 min of sonication.

In a second experiment combinations of vigorous shaking and sonication were tried (Figure 3-5). One min of mixing, 2 min of sonication and an additional min of shaking yielded the greatest number of colonies, while if the time for sonication was increased, the number of colonies was reduced. Again, different times of mixing on the vortex did not yield significant greater colony numbers. Mixing the sediment sample on the vortex for 2 min yielded similar colony numbers as in the first (Figure 3-4).

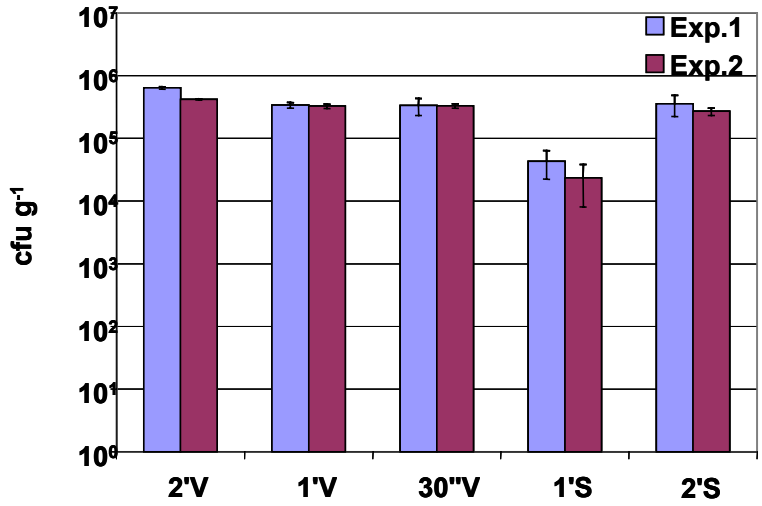


Figure 3-4 Detachment of Bacteria from Sediment Particles I. Sediment samples were mixed with PBS Buffer in a 1:1 (w/v) ratio and spread on R2A agar medium in serial dilutions and in triplicates. On the x-axis "minute" and "second" are abbreviated with their navigational pendants, "V" stands for vortex treatment and "S" for sonication (e.g. "2'V" means that the sample was shaken on the vortex for 2 min). The blue and purple bars represent results from two separate however identical experiments. Although plated in several dilutions, merely the results from the 10^{-3} dilution are shown for statistical comparability (also see chapter 2.8.7). Cfu counts at other dilutions, however, yielded similar results. The standard deviation from triplicate plating is shown by error bars.

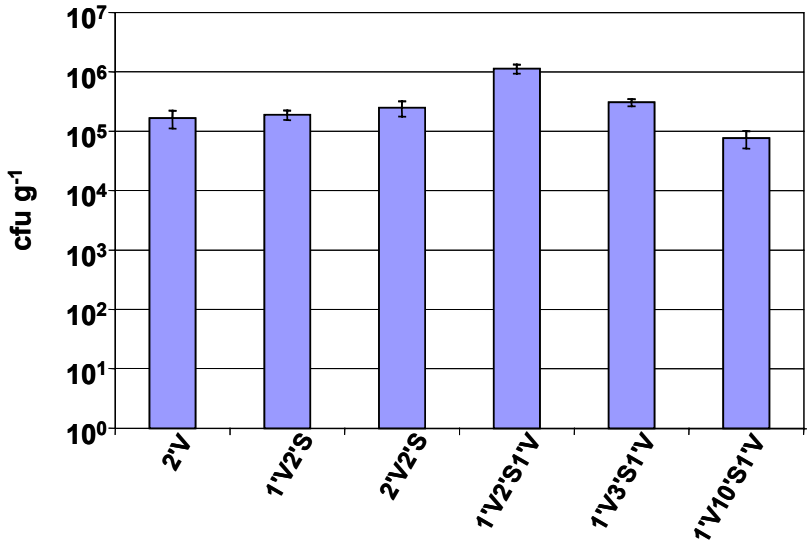


Figure 3-5 Detachment of Bacteria from Sediment Particles II. Since the duplicates from the first experiment had yielded similar colony counts, single treatments for the second experiment were performed, only. However, each treatment sample was spread in three serial dilutions and in triplicates. One treatment ("2'V") from the first trial was repeated and rendered similar colony counts as in the first experiment.

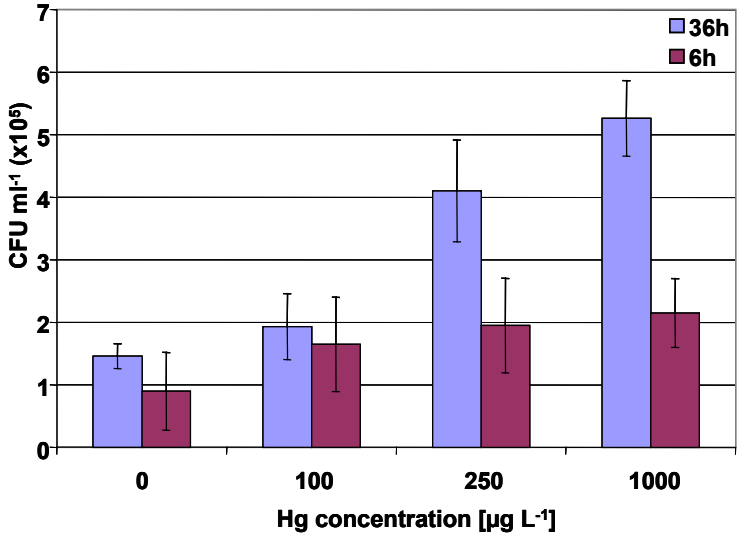
3.1.3.3 Effect of PMA on Bacterial Densities

The effect of different PMA concentrations on total bacterial densities was tested in a separate experiment using 250 ml Erlenmeyer flasks. Each flask was filled with 30 g of wet sediment from the Elbe River (corresponding to 10.6 g dry sediment) which was covered with 150 ml Elbe River water and supplemented with 0, 100, 250 or 1000 µg Hg (PMA) per Litre (corresponding to 0, 0.5, 1.25 and 5 µM Hg). Each mercury concentration was tested in duplicate flasks. The Erlenmeyer flasks were shaken gently at 120 rpm to avoid suspension of the sediment but to allow movement of the water phase. After 36 h the flasks were sampled and the total bacterial densitiy determined for sediment on R2A Agar (see section 2.2.1.4). When the experiment was repeated, flasks were sampled after merely 6 h and total bacterial densities determined both, for water and sediment. Unexpectedly, cell densities only differed by a factor five when amended with 1000 µg L^{-1} PMA compared with no addition of PMA. It was even more surprising to find cell density increase in the sediment with increasing mercury concentrations. This trend was confirmed in the second experiment although the effect was not as pronounced and total cell densities determined half of those in the first experiment. A possible explanation is the die-off of cells that were not resistant but released compounds which served as nutrients to the surviving cells, thus promoting their growth. After 36 h (and to a lesser extent after 6 h) the PMA-resistant bacteria could have transformed the mercury allowing for continued growth of sensitive cells. However, the differences were only minimal and within one order of magnitude.

In the water, cell densities were an order of magnitude lower than in the sediment. Here, the cell densities were decreasing with increasing PMA concentrations, although the trend was only minimal, with the density at 1000 µg L^{-1} mercury being not even half the density without mercury. The fact that less cultivatable cells were in the water while the cfu increased for the sediment supports the above suggestion that dead cells may have served as a source of nutrients for the remaining bacteria.

For the second microcosm experiment, when PMA was added to one of the microcosms in order to create a selective pressure, a concentration of 250 µg L^{-1} mercury (PMA) was chosen. In this preliminary experiment it was shown that PMA at this concentration did not eliminate the majority of the bacteria and did show a notable effect, in water and sediment.

(A):



(B):

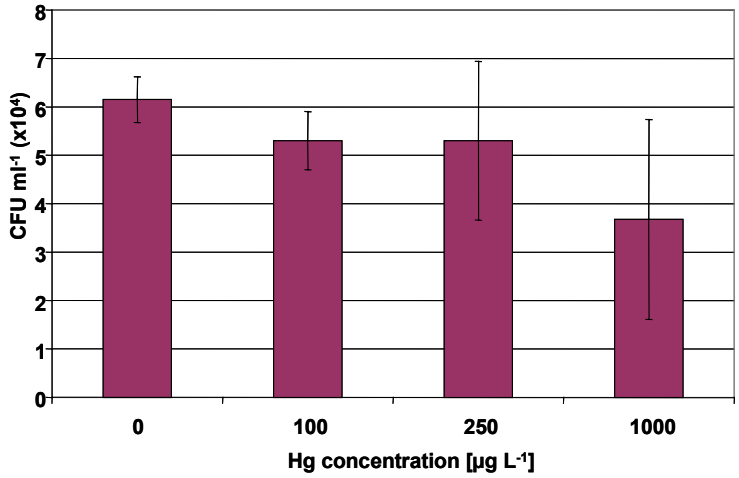


Figure 3-6 Effect of different PMA concentrations on culturable cell densities in sediment (A) and water (B). Slurry microcosms were incubated for 36 h or 6 h and the sediment (or water and sediment) sampled to determine total bacterial densities. While it was a surprise to find the density increase in sediment with higher mercury concentrations, at the same time total bacterial density decreased in water.

3.1.4 Survival of *Ps. putida* KT2442::mer73 in Stream Microcosms

In order to investigate the survival of the GEM *Ps. putida* KT2442::mer73 in stream water and sediment, three microcosms were operated in parallel. One was inoculated with the GEM (designated MCI), one with the parent strain *Ps. putida* KT2440 (MCII) and one remained uninoculated (MCIII) to obtain background data.

The microcosms were allowed to stabilize for 10 days prior to inoculation (recycling period) with recirculation of water at 1000 ml min^{-1}. For comparison, Eichner *et al.* (1999) operated

laboratory scale sewage plants which they allowed to equilibrate for 1 week before they started with their experiments. Schulz & Liess (2001) let their stream microcosms establish for three weeks before introduction of the (macroinvertebrate) organisms.

On day 10, the tap water inflow was opened and fresh water added to the microcosms at 15 ml min^{-1}. *Ps. putida* KT2442::mer73 was inoculated continuously into the system from day 10 on to yield GEM numbers in the water between 10^1 and 10^2 cfu ml^{-1} (Figure 3-7). From then on a slight decrease in total bacterial density was observed to day 55, the end of the experiment. Although rivers in nature do not encounter repeated flow of nutrients and/or bacteria, recirculation in this model was the best possible way to maintain a stable bacterial community without access to natural river water. The implementation of a recirculation also permitted much faster flow velocities.

The long-term inoculation of relatively small numbers of GEMs was performed to simulate a continuous leak or wash-out of GEMs into a river. Two weeks after the start of inoculation, a cell shock was performed, inoculating with the GEM to yield 2×10^6 cfu ml^{-1} to imitate a different scenario, namely the introduction of large numbers of GEM into a river, e.g. if bioreactor contents would accidentally be flushed. In the water, the GEM did not grow to higher cell numbers than introduced over the course of the experiment, neither by continuous inoculation of low cell numbers, nor through introduction of large cell numbers (Figure 3-7). In the sediment, a tenfold increase of GEM density could be observed over the time course of the experiment (Figure 3-8). However, bacterial densities were fluctuating and a longer observation period would have been necessary to confirm this trend.

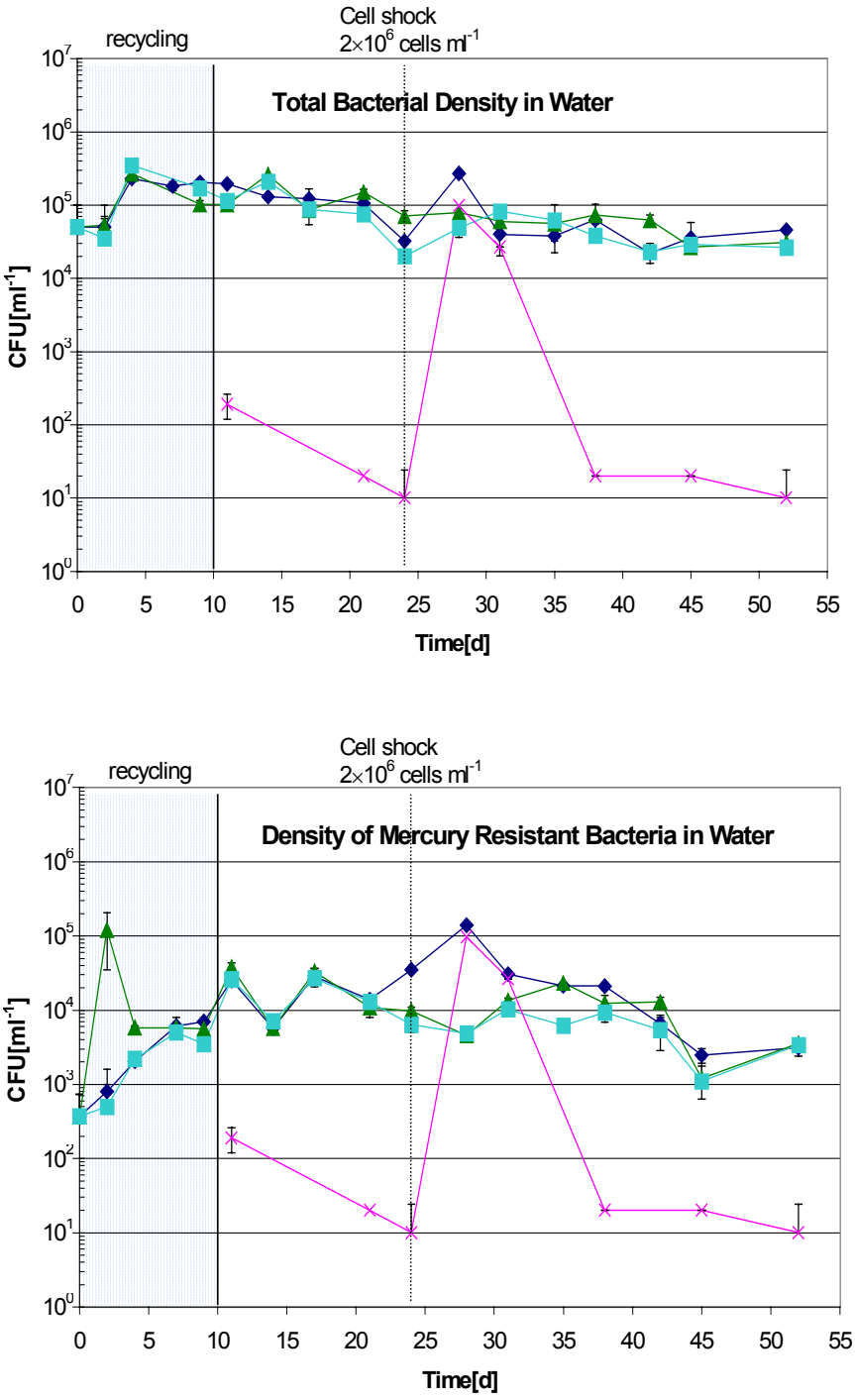


Figure 3-7 Microcosm Experiment I: Effect of the GEM on Densities of Total and Mercury Resistant Bacteria in the Water. [—◆—] Microcosm inoculated with the GEM (MCI), [—▲—] control microcosm that was inoculated with the parental wild type (MCII), [—■—] un-inoculated control microcosm (MCIII), and [—×—] GEM. Elbe water was recycled through the microcosms for 10 days before continuous addition of tap water and GEM or parent strain. At day 24 shock loads of the GEM were added to MCI yielding 2×10^6 cells ml^{-1}. For every sample bacterial densities were determined as cfu in triplicate. The standard deviation (s_{n-1}) is also shown in the figures, although sometimes masked by the symbols.

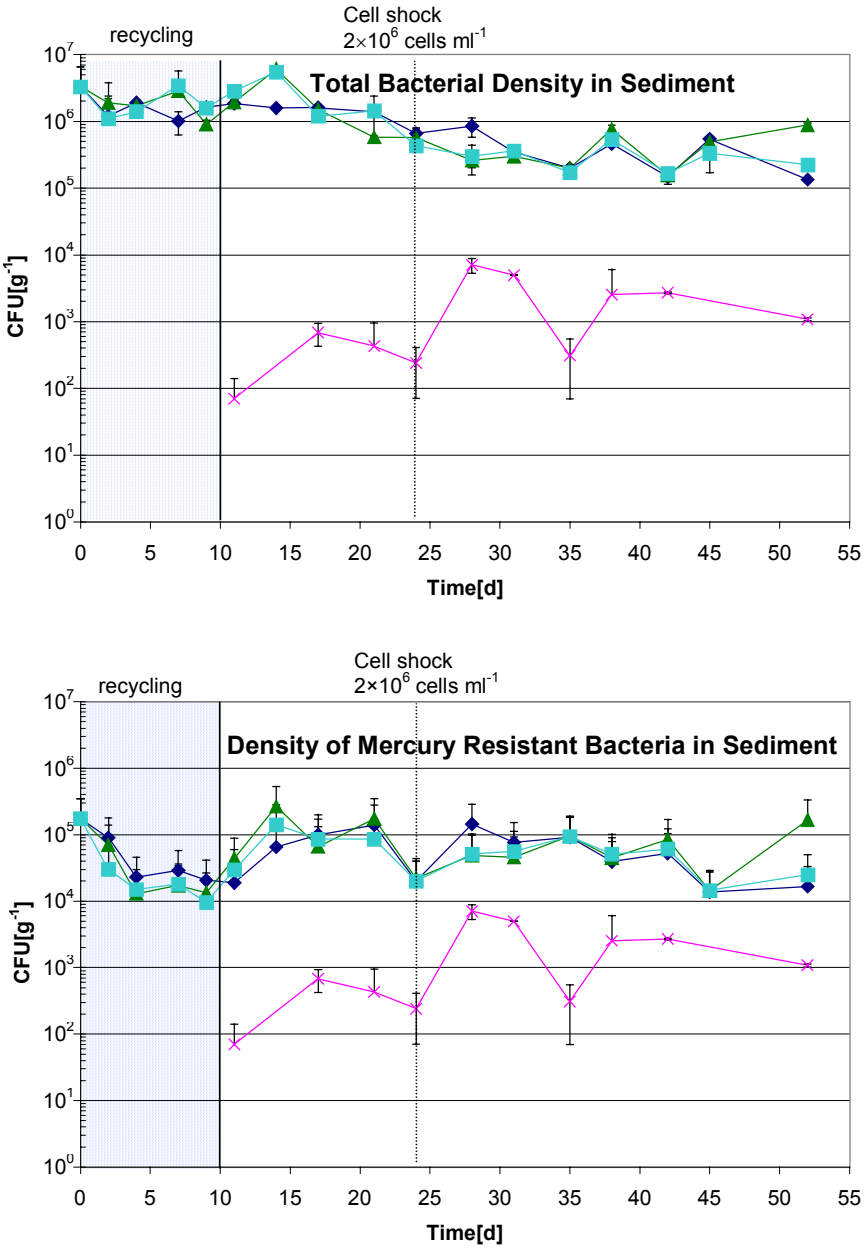


Figure 3-8 Microcosm Experiment I: Effect of the GEM on Densities of Total and Mercury Resistant Bacteria in the Sediment. [—◆—] Microcosm inoculated with the GEM (MCI), [—▲—] control microcosm that was inoculated with the parental wild type (MCII), [—■—] un-inoculated control microcosm (MCIII), and [—×—] GEM. Elbe water was recycled through the microcosms for 10 days before continuous addition of tap water and GEM or parent strain. At day 24 shock loads of the GEM were added to MCI yielding 2×10^6 cells ml^{-1}. For every sample the bacterial densities were determined as cfu in triplicate. The standard deviation (s_{n-1}) is also shown in the figures, although sometimes masked by the symbols.

3.1.5 Effect of the GEM on Total and Mercury Resistant Bacterial Densities

To determine an influence of the GEM on the cultivatable total or mercury resistant bacterial numbers, cfu were determined over the course of the experiment (Figure 3-7 & Figure 3-8).

Total bacterial densities declined approximately 10fold over time, both in sediment and water. However, the same trend could be observed in all three microcosms (uninoculated and inoculated with the GEM or the wild type *Ps. putida* KT2440) and thus cannot be ascribed to the GEM. Mercury resistant bacterial densities remained stable after inoculation for about five weeks both in water and sediment before declining 5-10fold.

During the recycling period total bacterial densities remained stable in the sediment and increased 5fold in the water. In addition, mercury resistant bacterial densities increased in the water from 3×10^2 to approximately 10^4 cfu ml^{-1} during this time period. At the same time mercury resistant bacterial densities decreased one order of magnitude in the sediment. These effects may be accredited to population shifts resulting from to the new constraints of the microcosm ("artefacts of enclosure", "succession and self-organisation", "bottle effect": Berg *et al.* 1999, Schäfer *et al.* 2000, Beyers & Odum 1993, Wagner-Döbler *et al.* 1992). With the cell shock on day 24, a brief increase of total and mercury resistant bacterial densities in water could be observed in the GEM inoculated microcosm. Mercury resistant bacterial densities were similar to those determined for the GEM, and after 8 days they had declined to densities found in the control microcosms. The same effect was seen for total bacterial densities in the water. In the sediment, densities of total and mercury-resistant bacteria remained rather unaffected by the cell shock, possibly because GEMs only penetrated into the top layer of the sediment after introduction.

3.1.6 Statistical Analysis of Total and Mercury Resistant Bacterial Densities

Figure 3-7 & Figure 3-8 suggest that total bacterial densities in all three microcosms were identical. Statistical analysis was performed to confirm this homogeneity between the microcosms (Table 3-1) by applying the t-test for paired observations (see 2.8.7; Sachs 1968). This t-test takes the differences of the bacterial densities of two microcosms and compares them to zero. If the two densities are equal (i.e. if the difference is close to or equal to zero), the resulting t-values are below the critical t-value. If the differences between the two microcosms are significant, the t-value will be greater than the critical t-value.

The t-values for total and mercury resistant cell densities in water and sediment confirmed that there was no difference in the bacterial densities between the three parallel microcosms.

Table 3-1 The t-values for the statistical analysis of the bacterial densities obtained for total and mercury reducing bacteria in the water and in the sediment. The critical t-value for a two sided t-test at a significance level of α=0.05 and 15 degrees of freedom (Total cfu in water) was ±2.131, for all other curves it was ±2.120 (α=0.05, df=16). Values below the critical t-value indicate equal bacterial densities that were significant.

t-values	MCI/MCII (+GEM/ +parent strain)	MCI/MCIII (+GEM/ uninoculated)	MCII/MCIII (+parent strain/ uninoculated
Total cfu in the water	0.357	0.719	0.635
Hg-resistant cfu in the water	0.231	1.619	1.478
Total cfu in the sediment	-1.094	-1.159	0.021
Hg-resistant cfu in the sediment	-0.883	1.018	2.012

3.1.7 Influence of the GEM on the Indigenous Bacterial Community

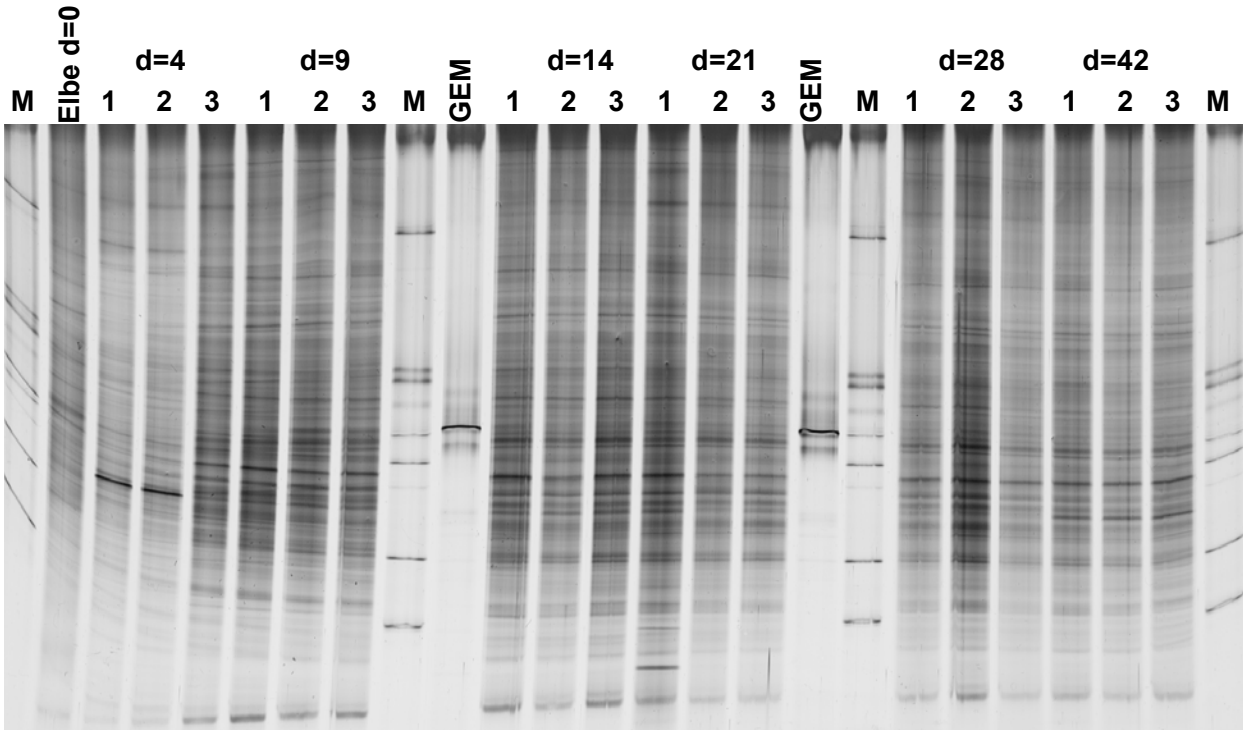
TGGE has proven to be a powerful tool for community monitoring after environmental changes (Muyzer and Smalla 1998). To detect whether the GEM would influence the indigenous microbial community in sediment and water, samples from the first microcosm experiment were profiled as patterns of amplified 16S rDNA fragments on thermogradient polyacrylamide gels (Figure 3-9).

3.1.7.1 Changes in the Bacterial Community in the Sediment

The sediment samples yielded an extremely complex profile. More than 50 bands could be detected with the naked eye. For comparison, Eichner *et al*. (1999) could find a maximum of 18 bands in activated sludge samples. The TGGE 16S rDNA profile from soil presented by Engelen *et al*. (1998) contained ca. 20-30 bands, and with DGGE Rasmussen *et al*. (1998) could visualise 25 vs. 50 16S rDNA bands for mercury contaminated soil *vs*. untreated soil.

A small community shift could be observed as the sediment was transferred from the Elbe River to the microcosms (confinement effect, see above). Small differences between the microcosms could further be observed in the beginning before inoculation (d=4), which might well be within the experimental variability since they often occurred in all three samples from one date. Later, there were no differences between the microcosms detectable. The 16S rDNA band of the GEM ran in accordance with one community band. Since *Ps. putida* is a widespread organism, likely also to inhabit Elbe River sediment, it is not surprising that the community band could be observed on the gel before inoculation with the GEM.

(A):



(B):

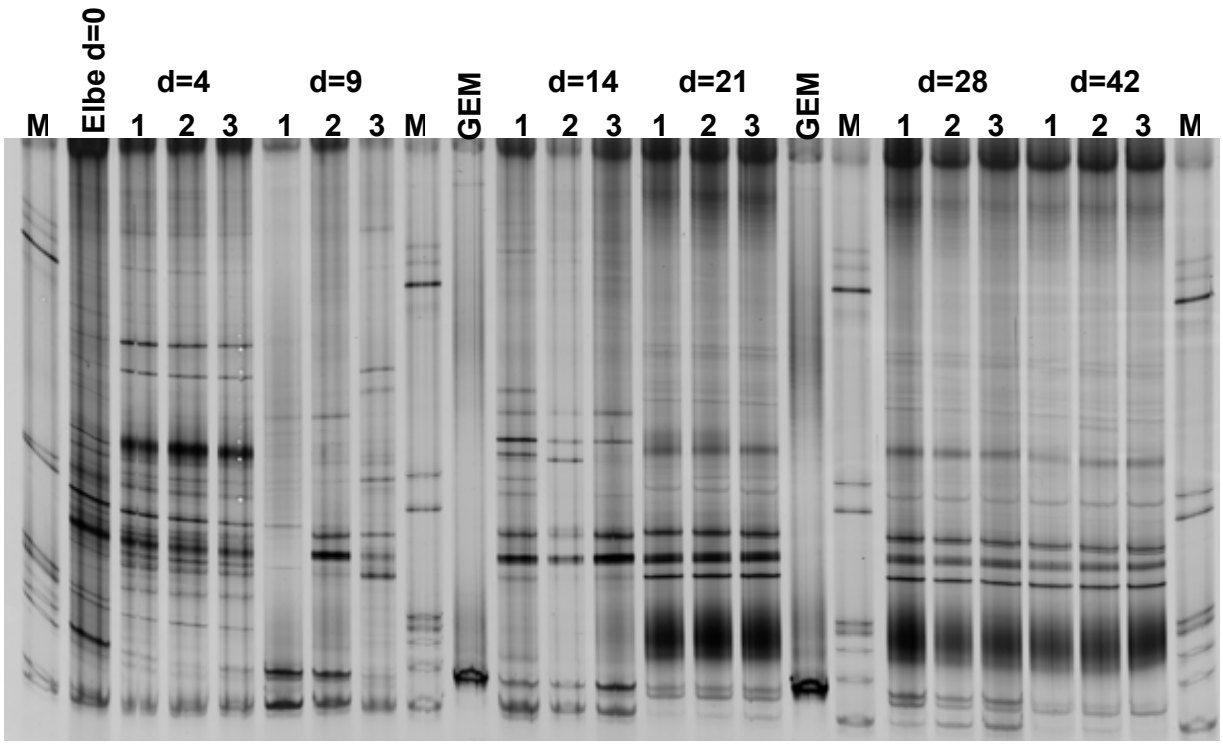


Figure 3-9 Bacterial Community Profiles based on TGGE of 16S rDNA fragments amplified from sediment (A) and water (B) over the time course of the microcosm experiment. A 16S rDNA marker (M) and the pure GEM were included in the gel for reference. Of the three parallel microcosms, one remained un-inoculated (1), one was inoculated with the parent strain *Ps. putida* KT2440 (2) and one was inoculated with the GEM, *Ps. putida* KT2442::mer73 (3). The days of sampling are indicated above the sample triplets.

3.1.7.2 Changes in Bacterial Community in the Water

Fewer bands could be amplified from the water samples. This was probably caused by differences in diversity, as discussed by Torsvik *et al*. (2002). A drastic decline in the number of bands after transfer of the samples to the laboratory could be observed with more than 30 bands in lane 1, representing the undisturbed water community based on 16S rDNA. The complexity of the pattern was reduced over the course of the experiment to approximately 20 bands of which some became very dominant. Differences between the three microcosms were undetectable on day 4 (before inoculation) and on days 21 - 42 (after inoculation). Pronounced differences were, however, observed on day 9 (before inoculation) and on day 14 (after inoculation). This was also the time of a reduction in diversity from 30 to 20 bands, caused by increased growth of some phylotypes and disappearance of others. Thus, differences in community profiles may have been caused by differences in the succession process, although the profiles eventually converged on day 21. The 16S rDNA band from a pure culture of the GEM ran in accordance with a community band that may have been derived from the GEM or from a wildtype *Ps. putida* strain since it was already present before inoculation.

3.1.8 Gene Transfer in Stream Microcosms

Gene transfer to an isogenic recipient strain in a simulated river environment was investigated in a second microcosm experiment including the influence of a cell shock and selective pressure (Figure 3-10). Three microcosms were run in parallel of which one (MCI) was inoculated both with the donor (*Ps. putida* KT2442::mer73) and the recipient (*Ps. putida* KT2442::Tc). Phenyl mercuric acetate (PMA) was added to the inflowing tap water to a final concentration of 250 μg L^{-1}. The second microcosm (MCII) was also inoculated both with donor and recipient but did not contain any mercury, and the third (MCIII) microcosm was operated without the addition of donor and recipient and without mercury. During the experiments donor and recipient were introduced continuously from two chemostats to yield cultivatable inoculant densities between 10^4 and 10^6 g^{-1} sediment (wet weight). On day 48 a cell shock was performed in MCI and MCII with 10^8 donor cells ml^{-1} in the water column. On day 53 PMA was added to MCI to give a concentration of 10 mg L^{-1} mercury.

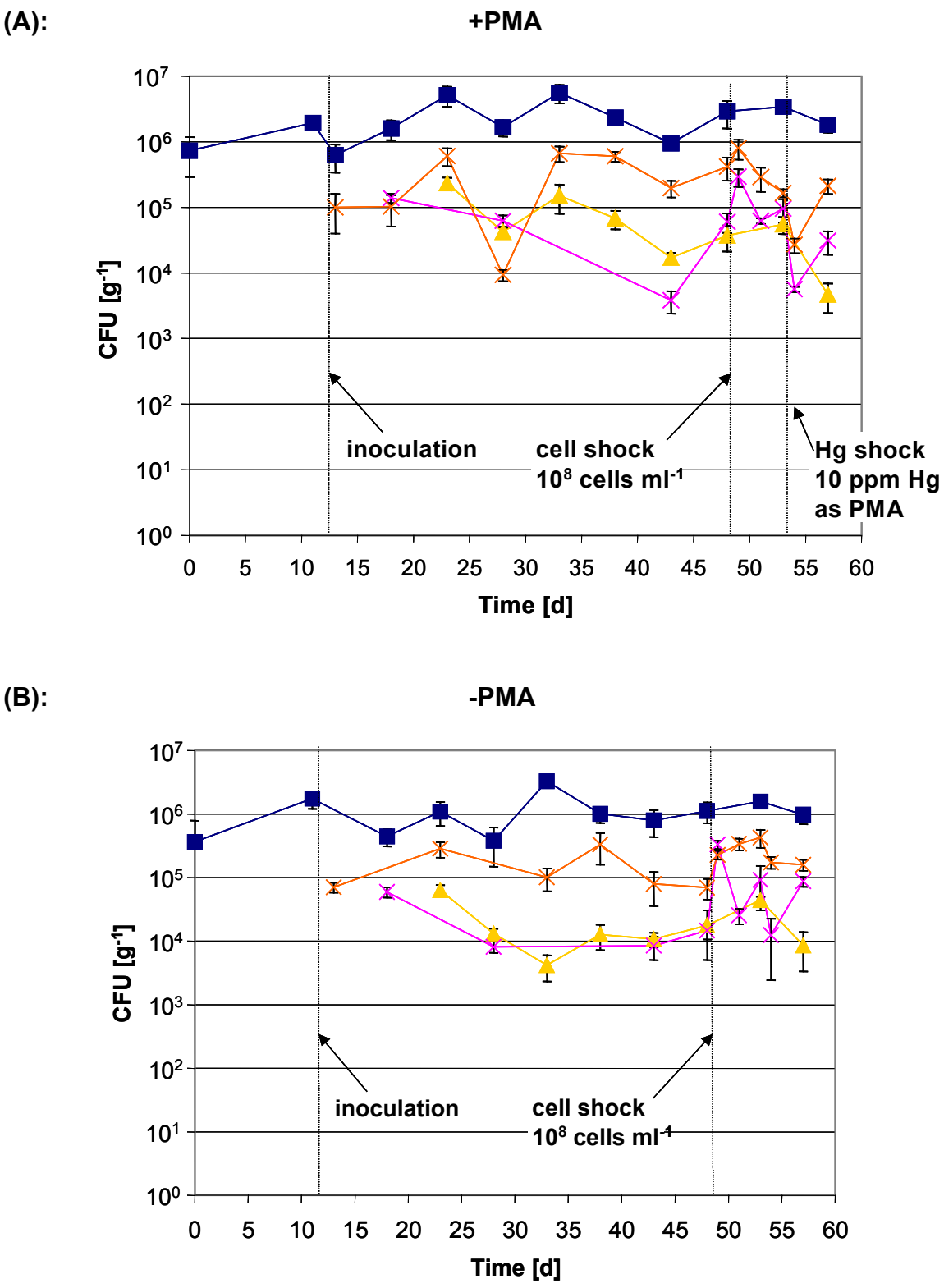


Figure 3-10 Gene Transfer Experiment. Bacterial densities in the sediment of microcosms MCI (A) and MCII (B), both were inoculated with the donor *Ps. putida* KT2442::mer73 and the recipient *Ps. putida* KT2442::Tc. MCI (A) was in addition amended with PMA to create a selective pressure. Total bacterial densities are depicted with a square, mercury resistant micro-organisms with a triangle, and donor and recipient are represented by a pink, and an orange star, respectively.

Total bacterial densities in MCI (+PMA, inoculated with the GEM and the parent strain) and MCII (-PMA, inoculated with the GEM and the parent strain) were approximately 10^6 per g of sediment over the duration of the experiment (57 d) and slightly higher in MCI (but well below 10^7 per g). The control microcosm MCIII also retained 10^6 cfu g^{-1} over the course of the experiment (data not shown, for natural background see Table 3-2). This bacterial density of cultivatable bacteria lies within the range that has been found before in uninoculated aquatic sediment microcosms with sediments from the Rhine River, Germany (Pipke *et al.* 1992).

Table 3-2 Background levels of mercury and antibiotic resistant bacteria in Elbe River water and sediment samples. Samples were spread on agar plates directly after Elbe River sampling.

Growth medium	Bacterial density in water (SD[1)]) [cfu ml^{-1}]	Bacterial density in sediment (SD) [cfu g^{-1}]
R2A	9.00×10^3(0)	7.33×10^5(4.44×10^5)
R2A-PMA[2)]	bd	bd
R2A-Tc	bd	bd
R2A-Rif	bd	bd
M9[3)]	9.67×10^1 ($1,07\times10^2$)	1.95×10^3 (2.27×10^3)
M9-PMA[4)]	bd	bd
M9-Tc	bd	bd
M9-Rif	bd	bd

bd: below detection

[1)]SD= standard deviation from triplicate plating

[2)]5 and 10 ppm mercury

[3)]supplemented with 10 mM benzoate as sole carbon source

[4)]1 ppm mercury

Mercury resistant bacteria both in MCI and MCII were mainly made up of the donor. In the control microcosm (MCIII), mercury resistant bacteria rendered between 10 and 100 cfu g^{-1} only (data not shown, for natural background see Table 3-2) as determined on R2A agar with 5 ppm mercury (PMA). For soil much higher frequencies have been determined (Smit *et al.* 1998). However, others have found mercury resistant cell densities in soil (Ranjard *et al.* 2000) or marine sediments (Rasmussen & Sørensen 1998) within the same range.

With the above assumption, that determination of mercury resistant bacteria did in fact yield donor numbers, the sparse data points for donor density can be completed and were between 10^4 and 10^5 ml^{-1} in MCI (+ PMA), i.e. up to one order of a magnitude greater than in MCII

(no mercury). A possible explanation is a competitive advantage of the mercury resistant donor strain compared with the natural population under selective pressure.

Recipient numbers were determined to be an order of magnitude higher compared with donor numbers in both, MCI and MCII despite identical treatment with regard to cultivation and inoculation to the microcosms. This was a consequence of different cell densities in the chemostats in favour of the recipient (10^8 cfu ml^{-1} vs. cfu 10^7 ml^{-1}). Rochelle *et al.* (1989) found highest frequencies for conjugal gene transfer of a mercury encoding plasmid from a mixed natural suspension of epilithic bacteria to *Pseudomonas sp.* at donor/ recipient ratios of 1.2×10^{-1} and 1.7×10^{-3}, therefore it may have been favourable for gene transfer to occur to have a surplus of the recipients. In the control microcosm recipient selective plates showed a background of between 10^2 and 10^3 cfu g^{-1} (for bacterial background levels see Table 3-2). However, recipient cell densities in the inoculated microcosms were generally two to three orders of a magnitude higher, thus the background did not affect the results

Transconjugant colonies were not found for the duration of the experiment. On selective medium for false positives transconjugants colonies could sometimes be observed, especially if much sediment was plated out together with the supernatant. However, those bacteria failed to grow if transferred to fresh medium containing mercury and tetracycline.

3.1.8.1 Influence of a Cell Shock on Gene Transfer

The cell shock raised donor cfu densities temporarily but did not have any notable effect on gene transfer. No transconjugant colonies could be made out even under conditions of temporary donor surplus. It must be stated that recipient numbers were high throughout and that the surplus of the donor only lasted for one day maximum. Nevertheless, the donor/recipient ratio was altered from ca. 10^{-1} to nearly 1 until the end of the experiment. However, this had no effect on gene transfer and did not result in transconjugant colonies.

3.1.8.2 Influence of a Mercury Shock on Gene Transfer

The mercury shock was applied as a selective force for the microorganisms to acquire mercury resistance genes in order to survive. One day after the mercury shock both, recipient and donor densities had dropped by at least one order of magnitude, probably due to die-off as a direct consequence of the mercury shock. It was surprising to find the mercury resistant GEM density diminish to a similar extent as the non-resistant recipient. This could be explained by the high concentration of mercury added. Von Canstein *et al*. (2002a) demonstrated highest mercury transformation rates in chloralkali waste water at 10 ppm mercury. However, they used $HgCl_2$ while in this experiment PMA was added. Horn *et al.*

(1994) who constructed the GEM determined resistance levels towards PMA of up to 65 ppm. They did, however, not add the mercury until the liquid culture had reached midlog phase. They also used a medium containing citrate and casaminoacids which may have bound some of the bioavailable mercury.

Since the total exchange of water in the microcosm was accomplished within 14 h it is not surprising that cultivatable bacterial numbers recovered quickly. This included total bacterial density and mercury resistant density that were not determined until 4 days after the mercury shock. Until the end of the experiment no transconjugant colonies could be recovered.

3.1.8.3 Quantification of the GEM in Sediment Samples by Competitive PCR (cPCR)

Determination of bacterial densities by a cultivation-dependent method such as cfu measurements bears numerous sources for error as already described in 1.3.1. Therefore, an alternative approach that was cultivation-independent was pursued to quantify the GEM *Ps. putida* KT2442::mer73 determining its chromosome equivalents (Felske *et al*., 2001). The basis for this method was the elucidation of the flanking chromosomal sequences to the insertion site of the *mer* operon in the construct strain (see 3.3). Both, *Ps. putida* genomic DNA and *mer* operon DNA sequence can be found in many environmental samples. However, the chimerical DNA region consisting of one end of the integrated *mer* operon sequence and the flanking construct genomic DNA was specific for the GEM and hence useful for detection and quantification by PCR.

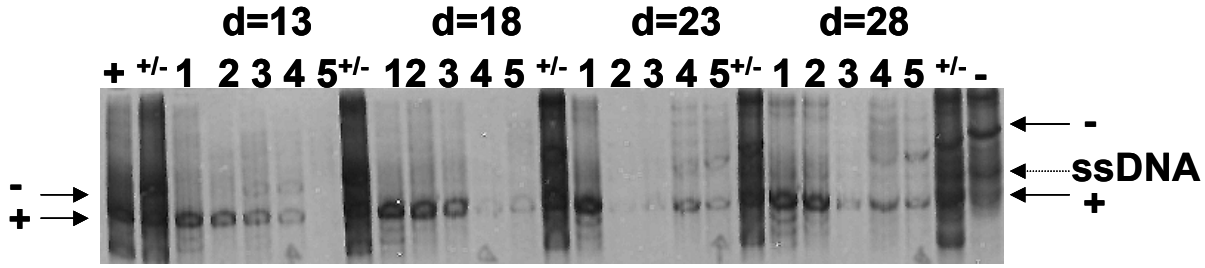


Figure 3-11 TGGE of PCR products from a competitive PCR co-amplifying a standard with total genomic DNA from MCII sediment samples. Standard concentrations added to the reactions were (1) 40, (2) 40/3 = 13.3, (3) 40/9 = 4.4, (4) 40/27 = 1.5, and (5) 40/81 = 0.5 fg. These correspond to (1) 4.6×10^7, (2) 1.5×10^7, (3) 5.2×10^6, (4) 1.7×10^6, and (5) 5.7×10^5 GEM chromosomal equivalents.

The primers used for the amplification were RECb and GC-RECp (Table 2-4) that yielded a fragment of 181 (142 + 39) bp in length. Besides the DNA sample from MCII (containing the GEM, -PMA), another template, the so called standard was added to the reaction mixture in known concentrations. This standard was almost identical to the GEM sequence to be

amplified, except for a single base, that however, drastically changed the melting behaviour of the product. The products of the two templates could therefore be separated in a thermo gradient polyacrylamide gel (Figure 3-11).

To calculate the corresponding chromosome equivalents from the TGGE signals, the molecular weight of one base pair which is approximately 650 g mol^{-1} was an important parameter. The size of the standard DNA (or GEM chromosomal template between and including the primer sites) was 159 bp which amounts to 1.0335×10^5 g mol^{-1} of standard DNA. In terms of standard molecule numbers (1 mol = 6×10^{23} standard molecules or chromosome equivalents), this corresponds to 5.8055×10^3 per femtogram of standard molecules (or 1.7225×10^{-19} g per molecule of standard or GEM chromosomal template between and including the primer sites). Standard and template signals of equal intensity could be found either in lane four (days 13 and 18) or in lane five (days 23 or 28). Lane 4 shows the reaction to which 8.6×10^3 standard molecules had been added while lane 5 depicts the reaction with 2.9×10^3 standard molecules. When translating this into chromosome equivalents per g sediment, the dilution added by the DNA extraction has to be taken into account: DNA from 0.5 g of sediment were extracted and resuspended in 100 µl of sterile, demineralised water of which 1 µl was added to the competitive polymerase chain reaction. Hence, lanes four and five represent respectively 1.7×10^6 and 5.6×10^5 GEM chromosome equivalents per g sediment. Interestingly, for these four sample dates, the cell density determined in this way was tenfold compared with the results obtained by the cultivation dependent measurements. However, it seems reasonable that the PCR also detected DNA of dead, or non-culturable cells of the GEM. Moreover, upon introduction to the microcosm a portion of the inoculated bacteria probably lysed, liberating their genomic DNA which may have been stabilised by sediment components (clay minerals, humic acids) for some time (Lorenz & Wackernagel 1994). In fact, the 142 bp DNA region necessary to obtain a signal in the PCR may have remained intact much longer than the full genomic molecule. Similarly, Felske *et al*. (2001) found a discrepancy between cfu and chromosome equivalents up to two orders of a magnitude for bioreactor effluents which they attributed to the wash-out of mainly old biofilm fragments harbouring dead cells or cells in no good condition that would not have been culturable.

Here, the results of only four sampling dates and only for sediment samples are shown. However, water samples and sediment DNA from different sample dates were submitted to cPCR but did not give (good) signals. Moreover, to confirm these results, lower standard

concentrations would have to be added since results here were mainly obtained for the lowest of the five standard concentrations.

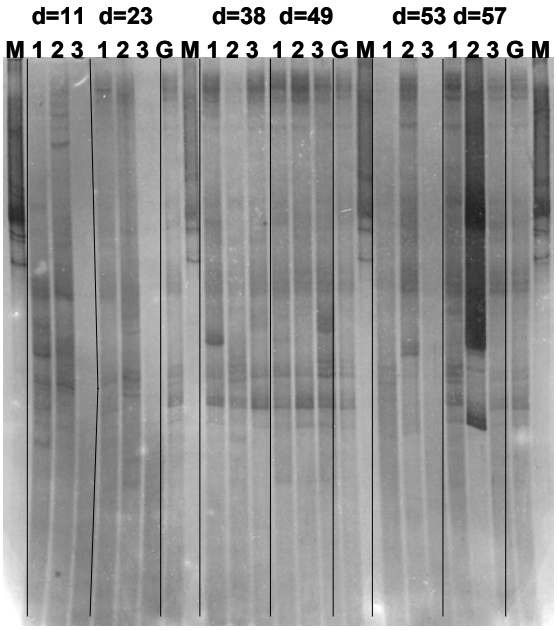
3.1.9 Mercury Resistant Community

The diversity of the *mer*A gene in the different microcosms was investigated for the second microcosm experiment, where PMA was added to one of the microcosms in order to create a selective pressure (Figure 3-12). Community profiles of all three microcosms are shown for each sampling date. Day 11 represents the last day of water recycling, before inoculation. Day 49 represents the day immediately after the cell shock. The sample was taken ca. 15 h after the cell shock, so that a full exchange of water had occurred since the addition of bacteria. A mercury shock was performed in MCI at day 53 with the addition of mercury yielding an initial mercury concentration of 10 ppm in the microcosm.

Neither in the water nor in the sediment samples the GEM signals could be found reliably. Although some diversity between the microcosms could be detected at all sampling dates (including the time period before inoculation and PMA amendment), the appearance and disappearance of bands seemed erratic and did not allow inferences.

The *mer*A PCR involves an initial touch-down PCR and further cycles including an annealing temperature as low as 46°C. The signals obtained gave rather diffuse bands in agarose gels (not shown) suggesting the presence of unspecific bands However, with a community DNA template the diffuse signal could also have been derived from a variety of specific *mer*A products of different lengths and was thus feasible.

(A):



(B):

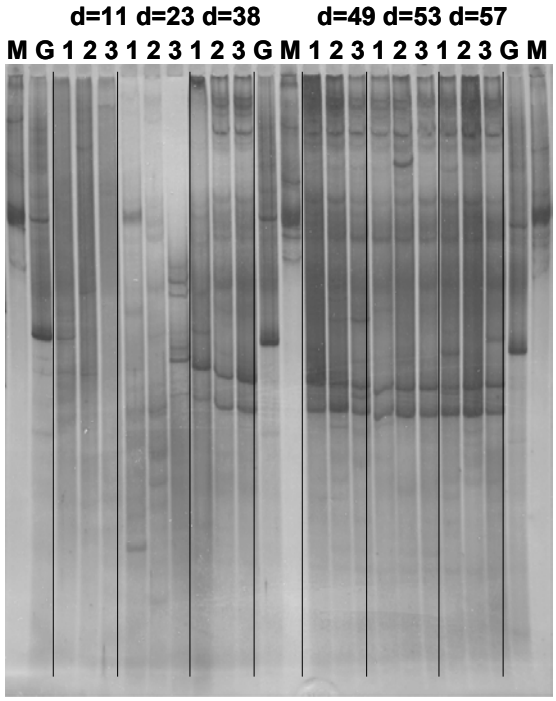


Figure 3-12 Community Profiles of *mer*A (mercuric reductase) Fragments in Stream Microcosm. Profiles ar shown for (A) water and (B) sediment. M = 16S rDNA size marker, G = pure culture of the GEM *Ps. putida* KT2442::mer73. Days of sampling were as indicated. Profiles for MCI, MCII and MCIII are shown in lanes labelled 1,2,3 respectively.

3.2 Construction of New Hg-Reducing, Fluorescent Strains

New mercury reducing, fluorescent strains were constructed by random Tn5-mutagenesis of *Ps. putida* KT2440 with a promoterless *mer*TPAB cassette consisting of the truncated, regulatorless *mer* operon of the *Serratia marcescence* plasmid pDU1358, followed by one of three different variants of a destabilized, FACS optimised GFP (Andersen *et al*. 1998). Figure 3-13 presents the cloning procedure schematically, while the respective steps are explained in further detail below (Figure 3-14, Figure 3-15, Figure 3-16).

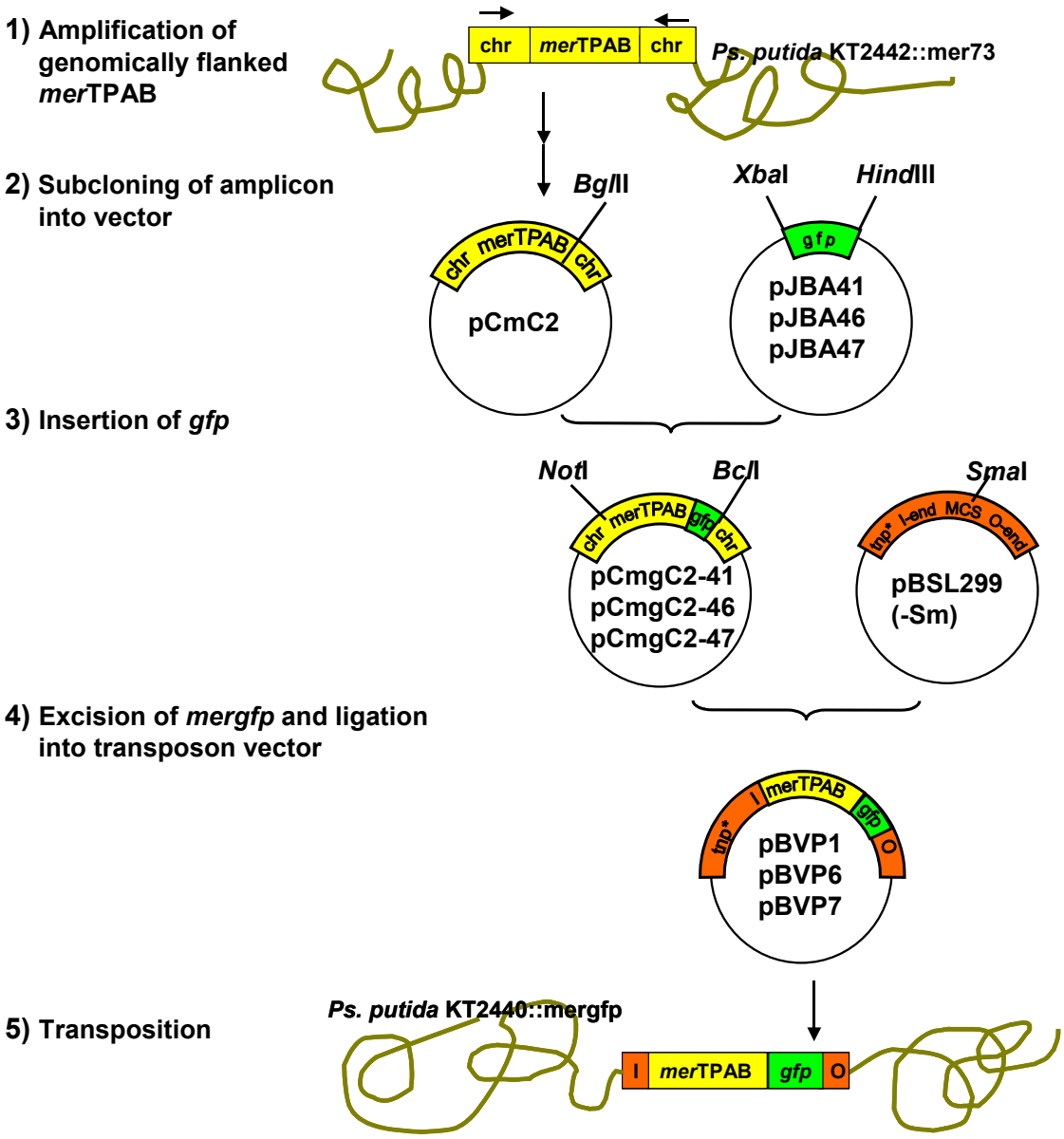


Figure 3-13 Cloning Strategy for New Constructs. (1) Initially, genomically flanked *mer*TPAB from *Ps. putida* KT2442::mer73 was amplified to take advantage of restriction sites that were upstream and downstream of the *mer*TPAB. (2) The amplicon was then subcloned into a plasmid vector, pCR TOPO BluntII, that was especially developed for the cloning of blunt end PCR products. The resulting plasmid was named pCmC1 (Figure 3-14). A shorter fragment consisting of the *mer* operon, still flanked by

chromosomal DNA was excised and subcloned into a pUC19 variant, yielding pCmC2 (Figure 3-15). The plasmid pUC19 is a commonly used cloning vector with an ampicillin resistance and a multiple cloning site within the *lacZ* gene that allows for blue-white selection. (3) The pCmC2 was cut open immediately downstream the *mer* operon and the *gfp* ligated blunt yielding pCmgC41, pCmgC46 and pCmgC47 (Figure 3-16). (4) Finally, the *mergfp* cassette was excised from these vectors and cloned blunt into the mini-Tn5-transposon vector pBSL299 (Alexeyev *et al.* 1995) that was prior deprived of its streptomycin resistance to prevent transposition of the antibiotic resistance. The resulting plasmids were named pBVP1, pBVP6 and pBVP7 (Figure 3-17). At last, (5) the mini-Tn5-transposons were transferred into *Ps. putida* KT2440 in three biparental matings to create the constructs. The most important restriction sites are shown in this figure. See text for further information.

3.2.1 Subcloning of the Mercury Resistance Operon

A region of the *Ps. putida* KT2442::mer73 genome was amplified (UPforII & DOWNrev, Table 2-4) generating a DNA fragment that encompassed the pDU1358 *mer*TPAB-operon flanked by *Ps. putida* KT2442 genomic DNA, 338 bp upstream and 136 bp downstream the insertion, respectively. The amplification was carried out with *Pfu* Taq DNA Polymerase (Stratagene, La Jolla, USA) which has retained its 3'-5' exonuclease activity, excising bases that have mistakenly been incorporated during strand extension. The amplicon was subcloned into pCR TOPO BluntII to yield pCmC1 and transformed into TOP 10 One Shot competent *E. coli* cells that were recommended by the manufacturer to be used with the pCR TOPO BluntII (Table 2-1). The plasmid vector pCR TOPO BluntII has especially been developed for cloning of blunt PCR products. Instead of performing a typical over night ligation prior to the transformation, the blunt end PCR amplicon is joined with the vector by a topoisomerase I that is covalently bound to the 3' end of the linearized vector. This takes no more than five minutes after which the vector can immediately be transformed into competent *E. coli* cells.

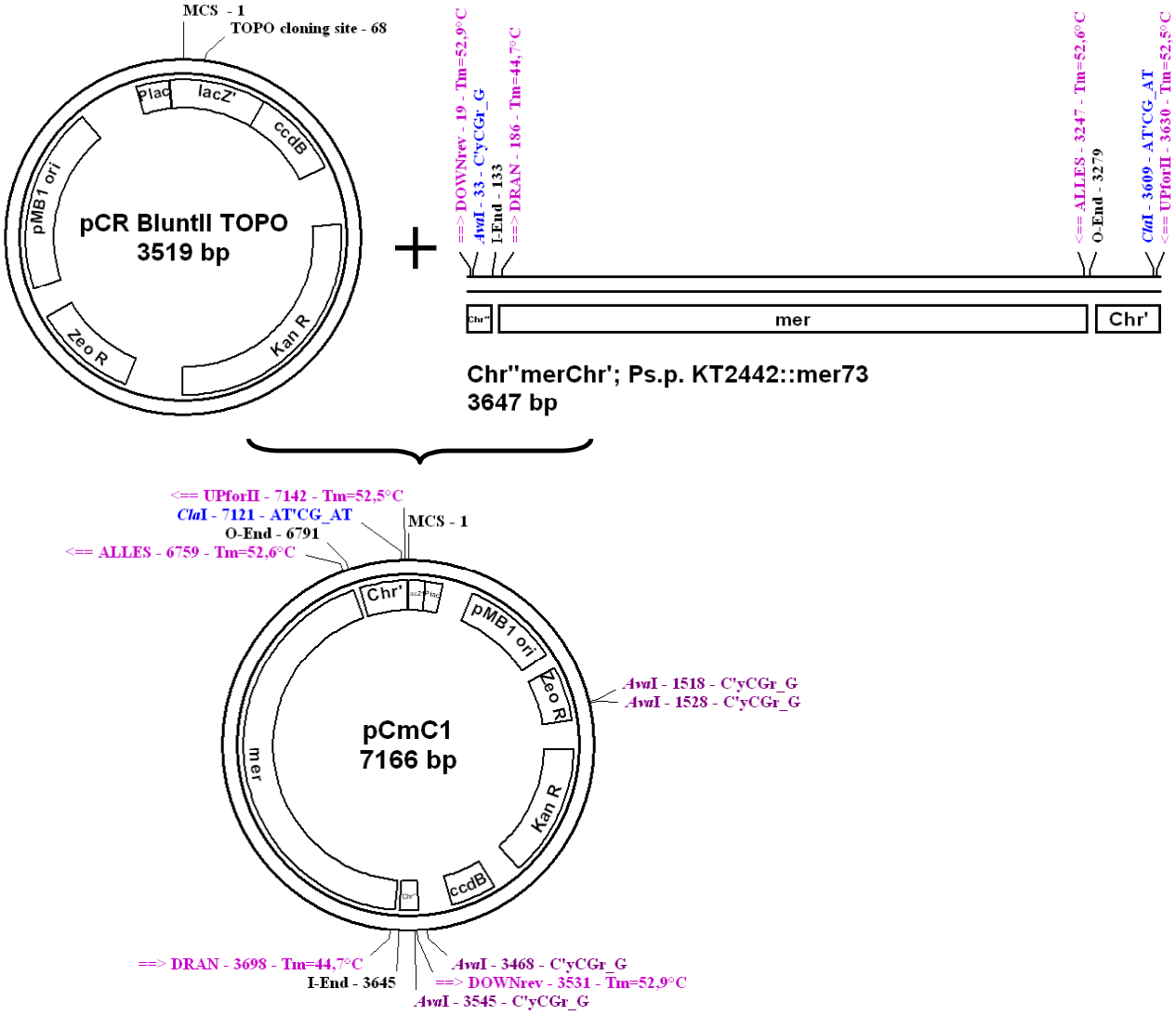


Figure 3-14: Cloning of pCmC1. *Ps. putida* KT2442::mer73 genomic DNA served as template for a PCR yielding a fragment that consisted of *mer*TPAB flanked by *Ps. putida* KT2442 chromosomal DNA. This amplicon was inserted into the TOPO cloning site of pCR BluntII TOPO. I- and O-ends of the previous transposition are shown as well as important recognition sites for restriction endonucleases and for primer annealing including their position on the plasmid. While the primer pair UPforII/DOWNrev was used for the generation of the fragment, the primer pair ALLES/DRAN marks the very ends of the *mer*TPAB, amplifying the entire operon. MCS = multiple cloning site. Tm = melting point for the associated primer.

The resulting plasmid pCmC1 was digested with *Ava*I and *Cla*I, rendering a fragment with 310 bp of genomic DNA upstream and 104 bp downstream the *mer*TPAB insertion which was subcloned into *Acc*I and *Ava*I digested pUC19oriT. Since *Acc*I and *Cla*I are compatible overhangs, this was a possible conjunction. The plasmid vector pUC19oriT carries in addition to the ampicillin resistance and the *lac*Z an origin of transfer and can thus be transferred by bacterial strains carrying *tra*-functions or if these are provided in trans. Although not relevant

for this construction, for other applications the oriT can be of importance, for example to transfer the plasmid by conjugation to bacterial strains, such as *Pseudomonas*, which are not as easily transformed as *E. coli*. However, for this construction the resulting plasmid, pCmC2, was transformed into Epicurian Coli® Electroporation-Competent Cells (*E. coli* XL1 Blue; Table 2-1).

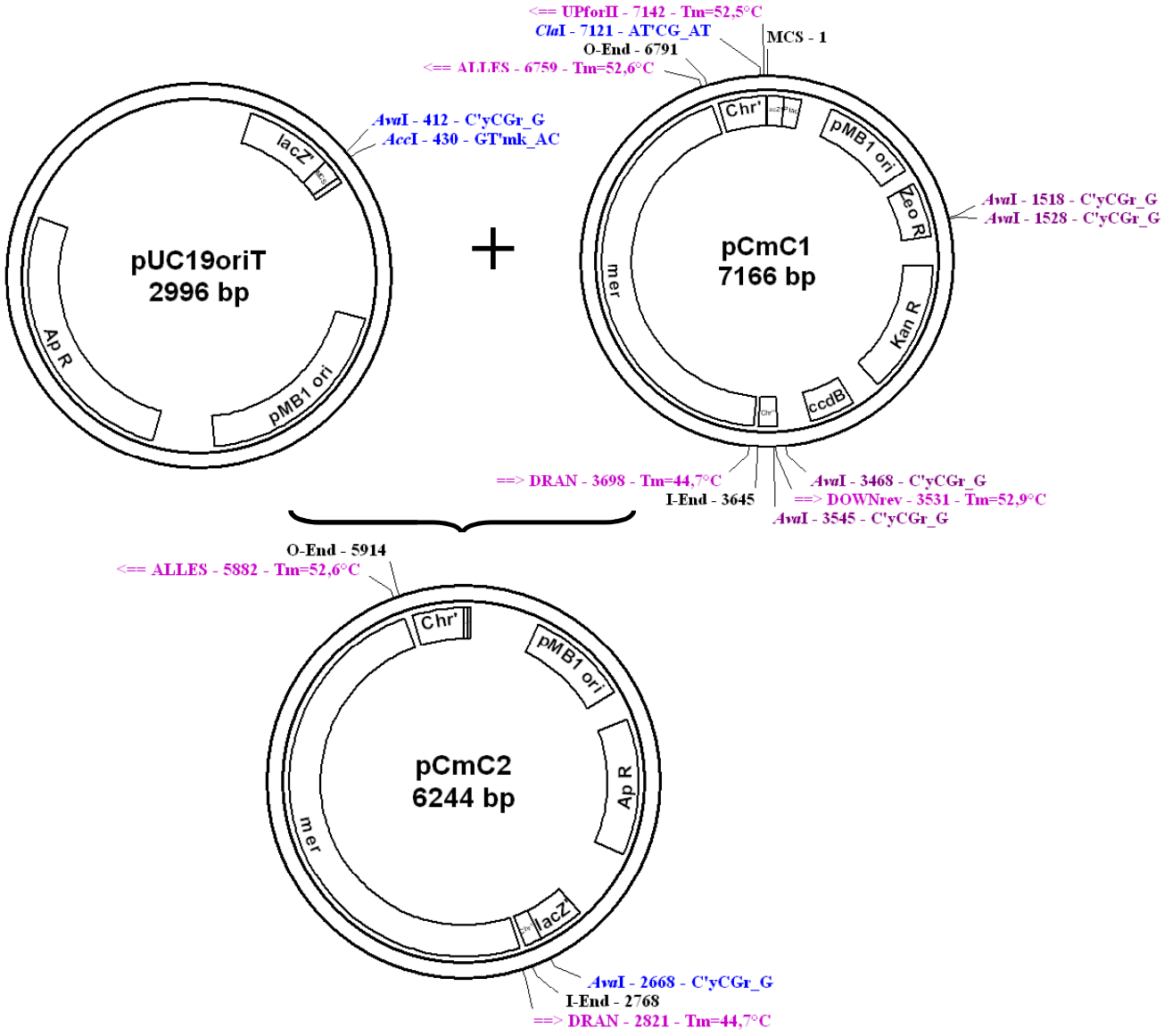


Figure 3-15 Cloning of pCmC2. *Ps. putida* KT2442::mer73 *mer* operon flanked by chromosomal DNA was inserted into pUC19oriT as *Aval*/*Cla*I fragment from pCmC1. I- and O- ends of *Ps. putida* KT2442::mer73 are shown, as well as relevant recognition sites for endonucleases and sites for primer annealing and their positions on the plasmid.

3.2.2 Creation of the *mergfp* Cassette

Three destabilized *gfp* variants with different half lives were used in this construction. The last three amino acids of the GFP from pJBA41 are LAA, this variant has a half life of 2½ min. The GFPs from pJBA46 and pJBA47 end with ASV and AGA respectively and exhibit half lives of 6 and >30 min. These GFP half lives were determined for *E. coli* (personal communication with M. Strätz and B. Andersen). The first two variants, -LAA and -ASV, were published in Andersen *et al*. (1998) where they were presented with half lives in *E. coli* of 40 and 110 min respectively. The last GFP version, -AGA, was not presented in the publication. Half lives, in *Ps. putida* KT2440, both for the -LAA and the -ASV variant were determined in the publication to be 190 min. However, in this work, the variants differed appreciably regarding their fluorescence due to differences in their transcriptional control.

The genes for the variants of destabilized GFP were excised from pJBA41, 46 and 47 (Andersen *et al*., 1998) with *Xba*I and *Hind*III and the cohesive ends filled with Klenow Fragment to create blunt ends. The *gfp* fragments were inserted into pCmC2 that had been prior digested with *Bgl*II, which lies immediately downstream the *mer* operon, and the resulting sticky ends filled with Klenow Fragment. Both, fragment and plasmid were ligated to yield pCmgC2-41, pCmgC2-46 and pCmgC2-47 (Figure 3-16) and transformed into *E. coli* JM110. For mercury reduction and GFP fluorescence to be coupled it was mandatory that *gfp* and *mer* operon be orientated in the same direction and transcribed from the same host promoter. Therefore, orientation of the *gfp* in the pCmgC2 variants were verified by a PCR that amplified the *mergfp* cassette (primers ALLES and GFPrev, data not shown, compare Figure 3-20).

3.2.3 Cloning of the *mergfp* Tn5 Minitransposon

The mini-Tn5 transposon that was used for the integration of the mercury-resistance genes and the gene for green fluorescence (*mergfp*) into the *Ps. putida* KT2440 genome, was derived from pBSL299 (Alexeyev *et al*. 1995). This transposon plasmid vector carried a streptomycin resistance gene between I- and O-ends which was excised with *Mlu*I and the plasmid subsequently re-ligated to yield pBSL299ΔSm. This resistance would otherwise have been transposed as well (see also 2.4.5.4).

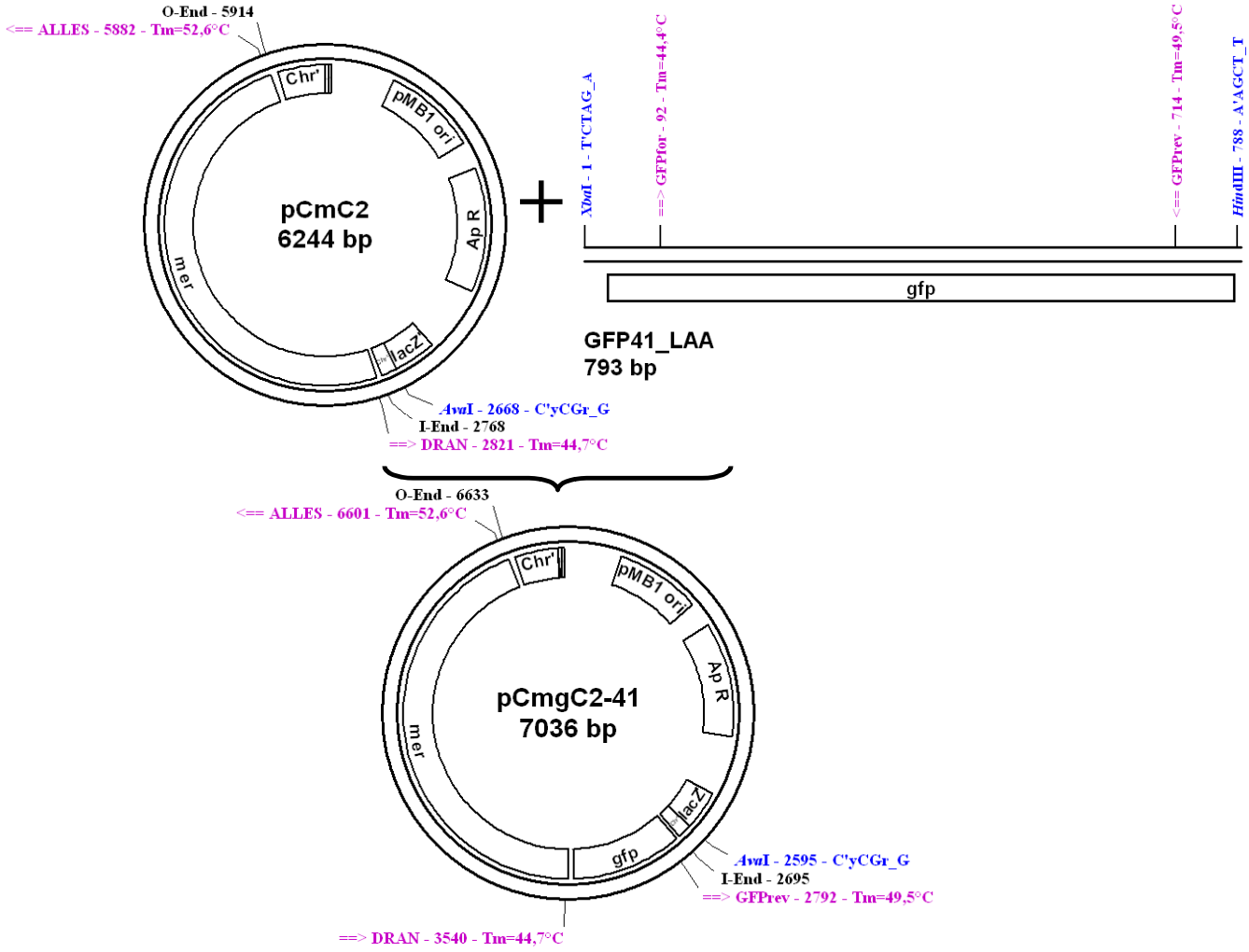


Figure 3-16 Insertion of *gfp* directly downstream *mer*. Cloning of the *mer-gfp* cassette into pCmC2 is shown for the most unstable GFP variant. Creation of the plasmid vectors containing the other two *gfp* variants was achieved likewise. I- and O-ends, relevant restriction sites and primers annealing sites are shown in the figure including their positions on the plasmid.

The *mergfp* fragment was obtained from pCmgC2-41, pCmgC2-46, and pCmgC2-47 with *Not*I and *Bcl*I and blunted with Klenow fragment. In most *E. coli* strains the *Bcl*I restriction cut site is methylated by the Dam methylase which transfers a methyl group from S-adenosylmethionine to the N^5 position of the adenine residue in the sequence GATC. It was therefore crucial that the plasmids be transformed into the Dam^- *E. coli* JM110 before they could be digested with *Bcl*I. The resulting blunt-end *mergfp* fragments with the three respective *gfp* variants were ligated into the *Sma*I site of the pBSL299ΔSm multi cloning site. Ultimately, the resulting donor mini-Tn5 transposons pBVP1, pBVP6 and pBVP7 were transformed into *E. coli* S17-1/λpir. This *E. coli* strain expresses the pir protein which is essential for replication of the transposon plasmids (see also 2.4.5.4).

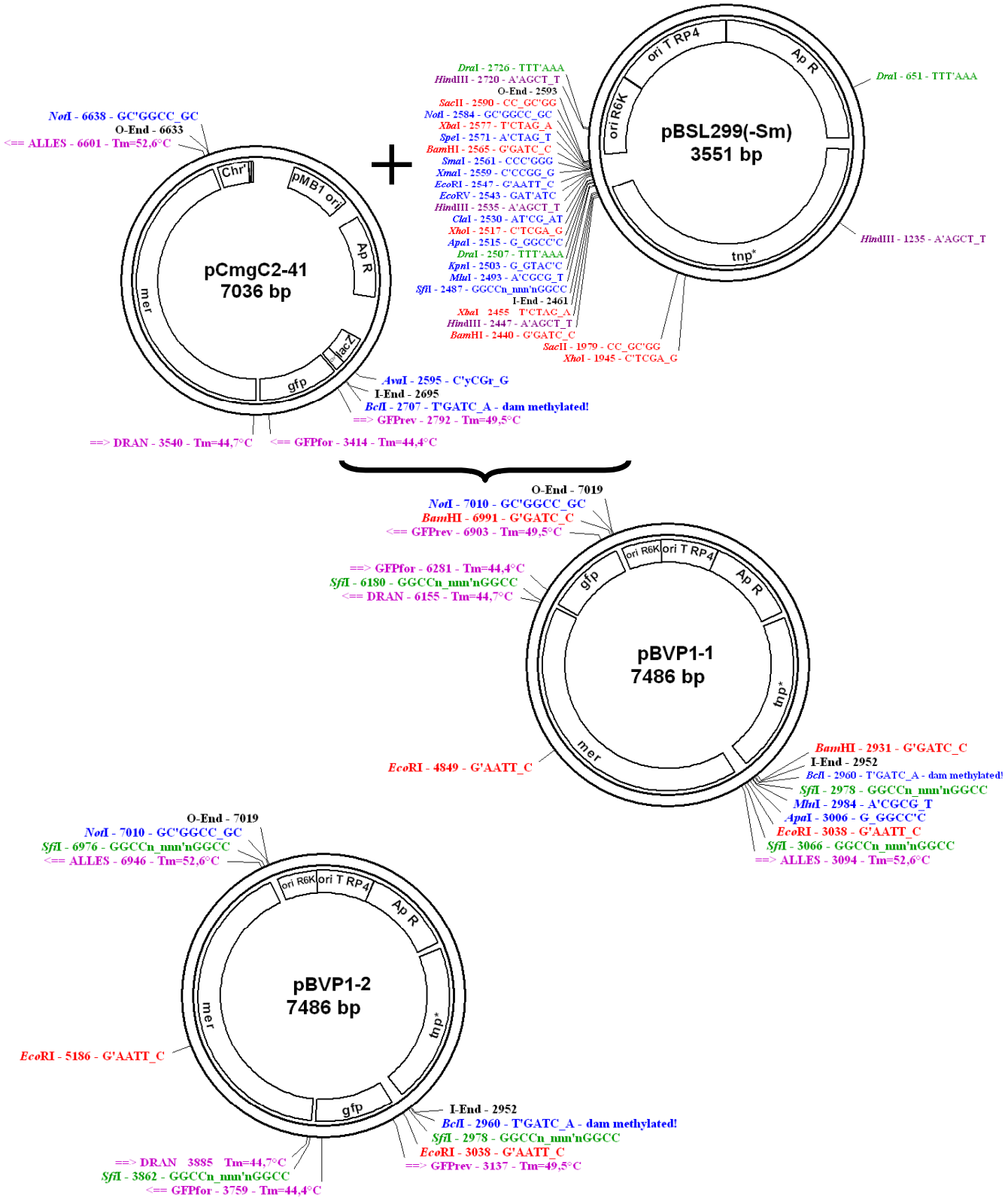


Figure 3-17 Cloning of *mergfp* mini-Tn5 transposons. The *mergfp* cassette from pCmgC2-41 was excised and ligated blunt into pBSL299ΔSm, resulting in two types of vectors with opposite orientation of the *mergfp* cassette (pBVP1-1 and pBVP1-2). The recognition sites of the pBSL299ΔSm multi cloning site are shown as well as relevant restriction sites and primer annealing sites of CmgC2-41. The *mergfp* cassettes of pCmgC2-46 and pCmgC2-47 were processed likewise yielding pBVP6 and pBVP7. See text for further information.

3.2.4 Selection of New Mercury Resistant *Ps. putida* Constructs

In three separate bi-parental matings pBVP1, pBVP6 and pBVP7 were transferred to the recipient *Ps. putida* KT2440. *Ps. putida* does not express the π-protein and is therefore incapable of replicating plasmids with a π-protein-dependent origin of replication such as the pBSL299 derived donor plasmids. In the cells in which transposition occurred the *mer-gfp*-cassette was integrated into the *Pseudomonas* genome (frequency of transposition of the pBSL minitransposons was tested by Alexeyev *et a1*. 1995 in *Klebsiella oxytoca* and was in the range of 10^{-5}). Mercury-resistant *Pseudomonas*-strains were selected on M9-minimal medium containing 10 mM benzoate as sole carbon source (which cannot be metabolised by *E. coli*) and 6 ppm Hg^{2+} (as $HgCl_2$).

From the selection plates 92 single colonies (30 each resulting from mutagenesis with pBVP1 and pBVP6, and 32 from mutagenesis with pBVP7) were picked and transferred to M9 minimal medium with 10 mM benzoate and 1 ppm Hg^{2+}. This is appreciably less mercury than was present in the selection medium. However, for the selection of transformants, a concentrated cell suspension was added to the agar plates after the mating, i.e. biomass containing proteins and amino acids with thiol groups that bind Hg^{2+} and thus decrease the bio-available mercury (Chang *et al*., 1993; Farrel *et al*. 1993). If less biomass is sequestering the mercury, lower Hg^{2+} concentrations are necessary to select for mercury-resistance in bacteria. Nevertheless, all transformants grew well with 1 ppm, therefore single colonies of all were transferred to solid M9 minimal medium with 3 ppm and 5 ppm Hg^{2+} (as $HgCl_2$).

While all transformants were still growing equally well with 3 ppm, differences in growth could be observed on plates with 5 ppm, i.e. some growing in thick streaks while others grew thin with single colonies only, suggesting cell death of a great number of bacteria. Although the number of colonies transferred from one plate to the next cannot be exactly quantified, transfer of all colonies was performed equally by sterilizing the inoculation loop in the Bunsen burner flame, then cooling it on the agar plate carefully before lifting a single colony and streaking it onto the fresh agar plate. Colony size was approximately 0.3-0.5 cm in diameter after three days (data not shown). To confirm the results obtained for the different construct strains, single colonies of all transformants were streaked on fresh plates with 3 ppm and 5 ppm Hg^{2+} once more (data not shown). Twenty strains with superior mercury resistance were selected based on these results.

For these 20 transformants, the mercury resistance level was in addition determined qualitatively by spreading overnight M9 (benzoate) cultures on solid M9 (benzoate) medium and adding one drop (10 µl) of a concentrated [10 g L^{-1}] $HgCl_2$ stock-solution to the middle of

the inoculated agar plate. After three days of growth, the diameters of the growth inhibition zones were determined and the plates photographed (Figure 3-18, Table 3-3).

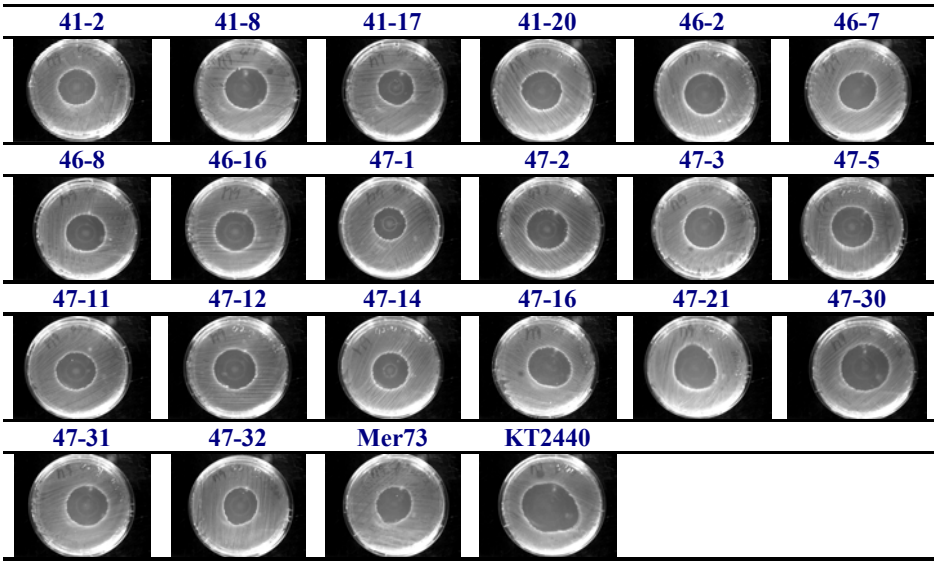


Figure 3-18 Inhibition Zones: Growth on M9 Agar with $HgCl_2$. Fresh liquid overnight cultures of the 20 construct strains were spread on solid M9 minimal medium with benzoate as carbon source, and 10 µl of a 10 ppm mercury stock solution ($HgCl_2$) were added to the centre of the plate. The agar plates were then incubated for three days at 30 °C and photographed.

Table 3-3 Growth on M9 Agar with $HgCl_2$: Diameters of Inhibition Zones. Seven of the new construct strains with the smallest inhibition zones are highlighted by bold print

Construct Strain (*Ps. putida* KT2440::mergfp)	Inhibition Zone-Diameter [cm]
Ps. putida KT2442::mer73	**3.1**
Ps. putida KT2440	4.3
41-2	**3.2**
41-8	3.6
41-17	**3.2**
41-20	3.6
46-2	3.6
46-7	**3.2**
46-8	**3.3**
46-16	3.5
47-1	**2.8**
47-2	3.5
47-3	3.4
47-5	3.6
47-11	3.4
47-12	3.5
47-14	**3.1**
47-16	3.6[1)]
47-21	3.6[1)]
47-30	4.0
47-31	3.5
47-32	**3.1[1)]**

[1)]Since the inhibition zone for these strains was distorted, measure was taken in both dimension of the ellipse and the average was given.

In another preliminary experiment conducted to characterize mercury resistance of the strains, 10 µl of liquid cultures (M9 + benzoate; OD=1.3) were added in serial dilutions to freshly prepared M9 (+ benzoate) agar plates containing 0, 3, 4, 5, 6 or 7 ppm of Hg ($HgCl_2$). Plates were incubated for three days and colonies counted.

After three days all construct strains showed colony densities on the plates ranging between 1 and 7×10^8 cfu ml^{-1}. All strains including the non-mercury-resistant *Ps. putida* KT2440 grew on agar plates containing up to 4 ppm Hg ($HgCl_2$), but not with 5 ppm Hg ($HgCl_2$) or higher concentrations.

The non-resistant *Ps. putida* KT2440 formed notably smaller colonies on mercury containing plates than on agar without mercury. Colony densities determined on agar plates without mercury varied between the strains by a maximal factor of eight (Figure 3-19, results shown for 10^{-5} dilution). Moreover, although colony densities were determined in triplicate, the volume of culture added to the plate was minimal (10 µl), yielding relatively few colonies and giving high standard deviations as indicated by the error bars.

However, if the attention is drawn to the decline in bacterial density at higher mercury concentrations, the construct strains behave differently. Strains that hardly showed a decline in colony densities with higher mercury concentrations were: *Ps. putida* KT2440::mergfp 46-7, 46-8, 47-1, 47-3, 47-8, 47-11, 47-14, 47-16, 47-31, 47-32, and the two mercury reducing strains *Ps. putida* KT2442::mer73 and *Ps. putida* KT2442::mer::gfp11. *Ps. putida* KT2440::mergfp 47-21 failed to grow at all in this experiment.

To validate these results statistically, the experiment would have to be repeated using higher volumes of culture added to agar plates. The sudden failure of all construct strains to grow with 5 ppm Hg ($HgCl_2$) cannot be fully accounted for, however, this has been observed before with other mercury resistant bacterial strains (personal communication with Wanda Fehr). A possible explanation that, remains to be confirmed would be that at this concentration (5 ppm Hg), all unspecific binding sites within the agar, from dead cells or the petri-dish rendering additional protection for the cells were saturated with mercury, so that a maximal number of mercury molecules was acting on the cells.

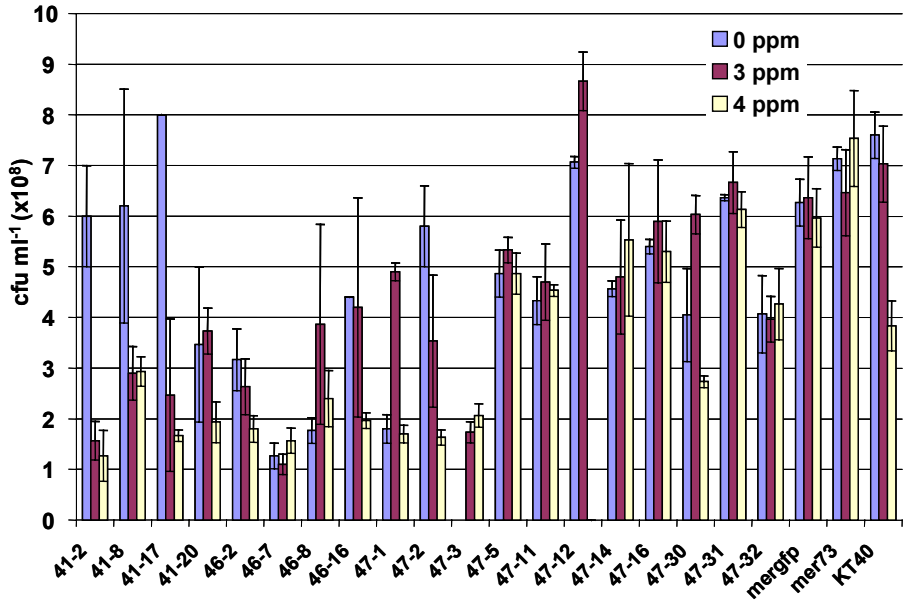


Figure 3-19 Decline in bacterial density with increasing mercury concentrations. Over night liquid cultures (10 µl) were pipetted in serial dilutions on agar plates with different mercury concentrations. Although, even without mercury, construct cultures did not yield the same densities, the decline in density allows differentiation of the strains with regard to their mercury resistance. Standard deviations from triplicates are shown by the error bars. See text for further discussion.

In order to verify purity of the transformants, M9 (+ benzoate) overnight cultures were streaked on LB-medium and colony morphology observed to allow differentiation of *Ps. putida* and *E. coli*. Colonies for all construct strains appeared to be *Pseudomonas*. However, on LB agar medium it was not possible to select for transformant *Ps. putida* strains, as both, donor and recipient were fluorescent and resistant to mercury. So purification of the *Pseudomonas* construct strains relied on frequent transfers and cultivation on M9 minimal medium with benzoate as sole carbon source which cannot be metabolised by *E. coli*.

A preliminary selection of the strains with the highest mercury resistance was undertaken with the information obtained with the above tests. These were observed with a fluorescence microscope (FITC filter: excitation at 475 nm, emission at 515 nm) to confirm GFP fluorescence. The strains showing best fluorescence and mercury resistance are listed in Table 3-5.

3.2.5 PCR Proof of *mergfp* Integration

Integration of the *mer-gfp* cassette into the genome of the recipient *Ps. putida* KT2440 was verified by PCR with primers binding to the 5'end of the *mer* operon and the 3' end of the *gfp*, respectively (ALLES & GFPrev, see Table 2-4), anticipating an amplicon of 3801 bp (Figure 3-20). One PCR-tube contained the reaction mixture, however, DNA template was

omitted in order to exclude signals due to contamination in the reaction mixture. Another PCR-tube was complemented with ca. 1 ng of the donor vector pCmgC41 as positive control. The genomic DNA of all selected constructs and of the positive control gave a PCR signal of the expected length. The negative control did not give a signal.

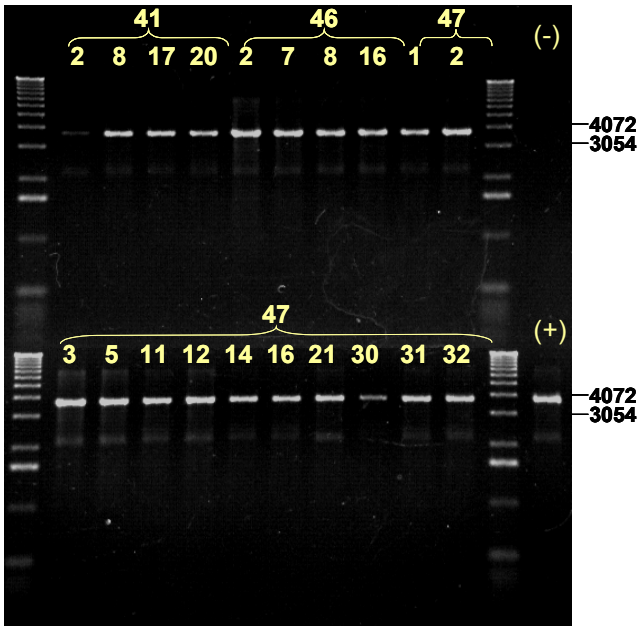


Figure 3-20 PCR proof of *mer-gfp* integration into the chromosomal DNA of *Ps. putida*. With chromosomal DNA as template the region between ALLES and GFPrev was amplified, expecting to yield an amplicon of 3.8 kb in length. The negative control did not contain any DNA template, the positive control contained pCmgC41 as template. The marker used was the 1 kb ladder (see Table 2-3).

3.3 Integration Sites and Genomic Environment in Ps. putida *KT2442::mer73 and in the New Constructs*

3.3.1 Revealing the Insertion Sites in *Ps. putida* KT2442::mer73 and in the New Constructs

Knowledge of the insertion site of a genetically engineered construct may help predict or explain the abilities (or disabilities) of the modified strain. The Directive of the European Parliament (2001/18/EC) considers good knowledge or description of the donor and recipient and the vector used in the construction as essential prerequisites for the use of modified organisms. Moreover, the integration site after transposon mutagenesis is unique for each construct and can form the basis for a detection with molecular techniques.

3.3.1.1 Sequencing the mer *Integration Site in* Ps. putida *KT2442::mer73*

To reveal the modified genotypes of both, *Ps. putida* KT2442::mer73 and the new constructs the sequence of genomic DNA immediately flanking the insertion site was elucidated. One method to amplify an unknown (genomic) sequence that lies next to a known DNA region is by using inverse primers that anneal to both ends of the known region (in this case the *mer* operon of *Ps. putida* KT2442::mer73, or *mergfp* cassette in the new constructs) but are directed outwards. The inverse PCR renders a signal if the ends of the template consisting of known (*mer* operon) and unknown sequence (genomic DNA) are joined (Figure 3-21). For more technical details see section 2.4.7.

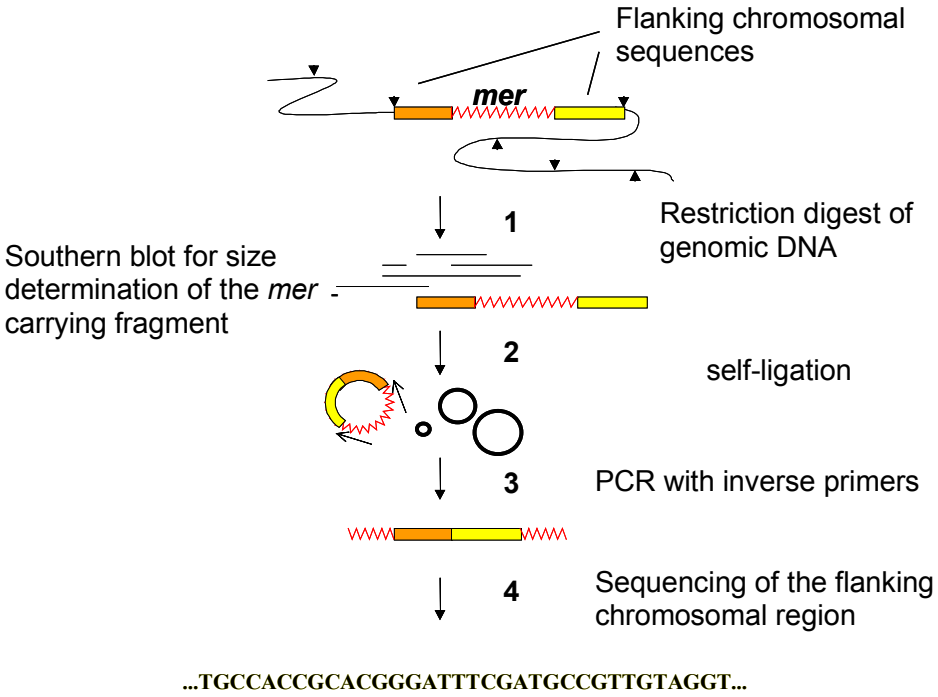


Figure 3-21 Inverse Polymerase Chain Reaction (redrawn from Ochman *et al.* 1988). (1) genomic construct DNA was digested with a restriction endonuclease. (2) The resulting fragments were self-ligated and (3) PCR performed with inverse primers. (4) The amplification signals were then sequenced. See text for further information.

Before the IPCR was performed, the size of the fragments carrying the *mer* operon was revealed in a Southern blot (Figure 3-22). *Ps. putida* KT2442::mer73 genomic DNA was digested with 12 different restriction endonucleases that did not cut within the *mer* operon, blotted onto nylon membrane and hybridised with a DIG-labelled probe against the transposed *mer* operon. Detection was achieved with a DIG-recognizing antibody-Alkaline Phosphatase conjugate and a substrate that was dephosphorylated. The resulting phenolate anion disintegrates to emit light that was detected on Kodak X-Omat x-ray film.

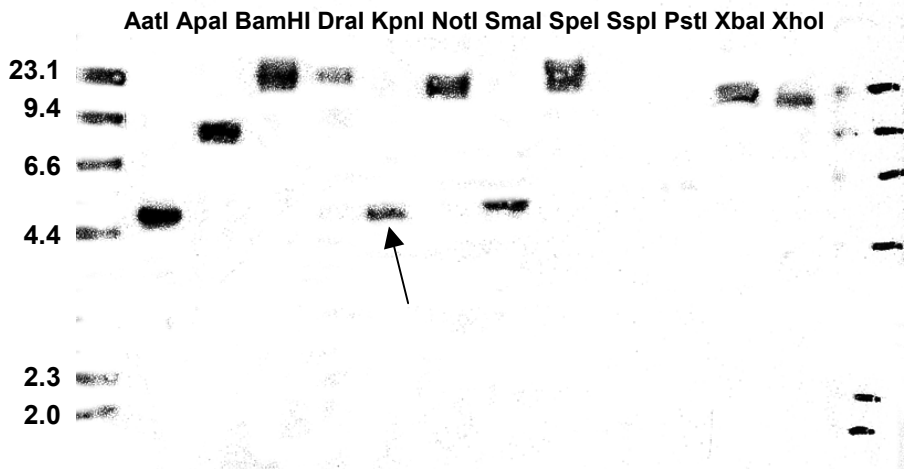


Figure 3-22 Southern Blot showing genomic Fragments containing the *mer* Operon. The arrow indicates the *Kpn*I digested fragment that gave a good signal in the IPCR. Marker specifications in the first and last lane of the blot are indicated as kilobases.

Fragments resulting from digests with the 12 enzymes of genomic *Ps. putida* KT2442::mer73 DNA were also self-ligated and IPCR was performed (Figure 3-23) with the primers TSRIF & LAIRT (Table 2-4). Knowing the size of the *mer* containing fragment (5.3 kb for *Kpn*I-digested chromosomal DNA), the length of the expected amplicon could be calculated by subtracting the length of the *mer* operon (3.1 kb). Out of 12 enzymes 6 yielded *mer*-carrying fragments greater 10 kb (see Figure 3-22), making it difficult to obtain a signal in the IPCR, as PCR efficiency decreases with very long amplicons. With the *Kpn*I digested, ligated chromosome as template, the primer pair was expected to give a signal at 2.2 kb (Figure 3-23). A bright signal of that size could indeed be obtained and was confirmed by a nested PCR with inverse primers annealing to the outermost regions of the *mer* operon (Table 2-4). A diffuse signal was obtained that was shorter than the first IPCR signal by approximately 300 bp, which is the length difference of the amplicons of the two primer pairs.

The bright 2.2 kb amplicon from the initial IPCR was excised from the agarose gel and sequenced with all four primers used in the IPCR and nested IPCR (TSRIF, LAIRT, SELLA, NARD; Table 2-4). Of the *mer*-genome hybrid sequence 174 bp have been deposited in GenBank under the accession number AJ251632.

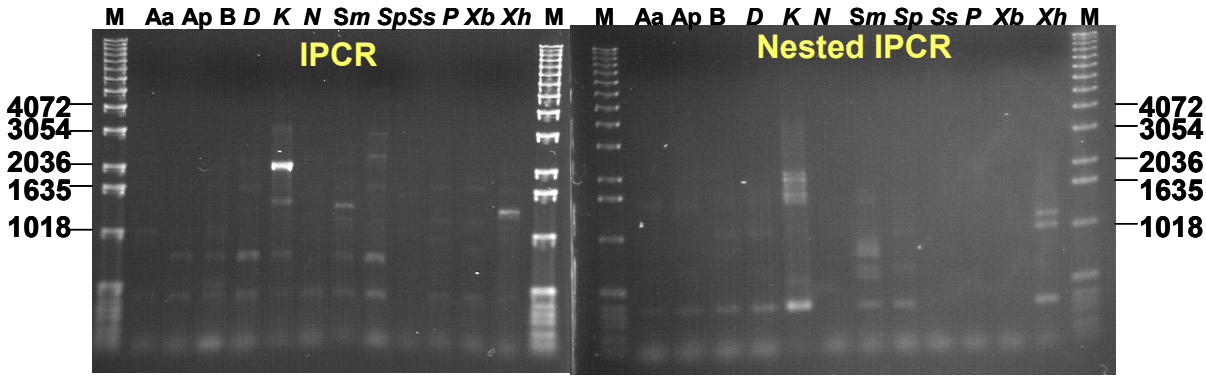


Figure 3-23 IPCR and nested IPCR. Re-circulated, chromosomal fragments of the GEM were templates for a PCR with inverse primers against the ends of the *mer operon*. A nested IPCR was performed to verify the signal(s) from the original IPCR. The size of the marker (M) bands is indicated as number of basepairs. The individual lanes show results from genomic fragments that were created with different endonucleases: Aa=*Aat*I, Ap=*Apa*I, B=*Bam*HI, D=*Dra*I, K=*Kpn*I, N=*Not*I, Sm=*Sma*I, Sp=*Spe*I, Ss=*Ssp*I, P=*Pst*I, Xb=*Xba*I, Xh=*Xho*I.

3.3.1.2 Sequencing the mergfp *Integration Site in the New Constructs*

How many *mergfp* cassettes have been transposed into the genomes of the construct strains remains to be elucidated for all twenty new constructs. Since transposition is relatively rare (10^{-5}) it is highly likely that the constructs have received only one cassette, however, the experimental proof by Southern Blot and hybridisation still needs to be furnished.

Inverse PCR with genomic construct DNA as templates was performed as described above but with the primer pairs TSRIF & LAIRT for the first IPCR, and SELLA & GFPrevinv (Table 2-4) for the nested IPCR (Figure 3-24). In contrast to the digests with many different enzymes of *Ps. putida* KT2442::mer73 DNA, for the new constructs restriction digests before IPCR were only carried out with *Kpn*I and not with a whole range of enzymes. *Kpn*I was chosen because *Ps. putida* KT2442::mer73 DNA had yielded a good IPCR signal. Moreover, Ramos *et al*. (2000) used *Kpn*I for localization of an insertion in a *Ps. putida* KT2440 construct by a similar approach. The enzyme seemed to yield an optimal number of fragments and fragment lengths with *Ps. putida* KT2440 DNA that appeared to be good templates for IPCR.

The second round IPCR did not only serve to confirm the right signal in the first PCR, in some cases the first IPCR yielded no or only weak products that however sufficed as templates and yielded stronger signals in the nested IPCR (46-2, 47-12, 47-30).

If no product was obtained even in the nested PCR (41-7, 41-20), this was probably due to a *mergfp* containing fragment that was too large to be amplified.

Sometimes, the first IPCR yielded shorter signals than the nested PCR (41-2, 41-8). For these constructs the signal of the first IPCR was below 1 kb, which is much shorter than would be expected since the ends of the *mergfp* cassette already constitute 1.2 kb of the expected amplicon and IPCR would yield an amplicon of that size even without adjoining genomic DNA. The signal was therefore probably unspecific or derived from a fragment template that was damaged having lost some of the ends of the fragment with the *mergfp* cassette. In these cases probably a much weaker signal that was not detectable in the first IPCR served as template for the nested PCR.

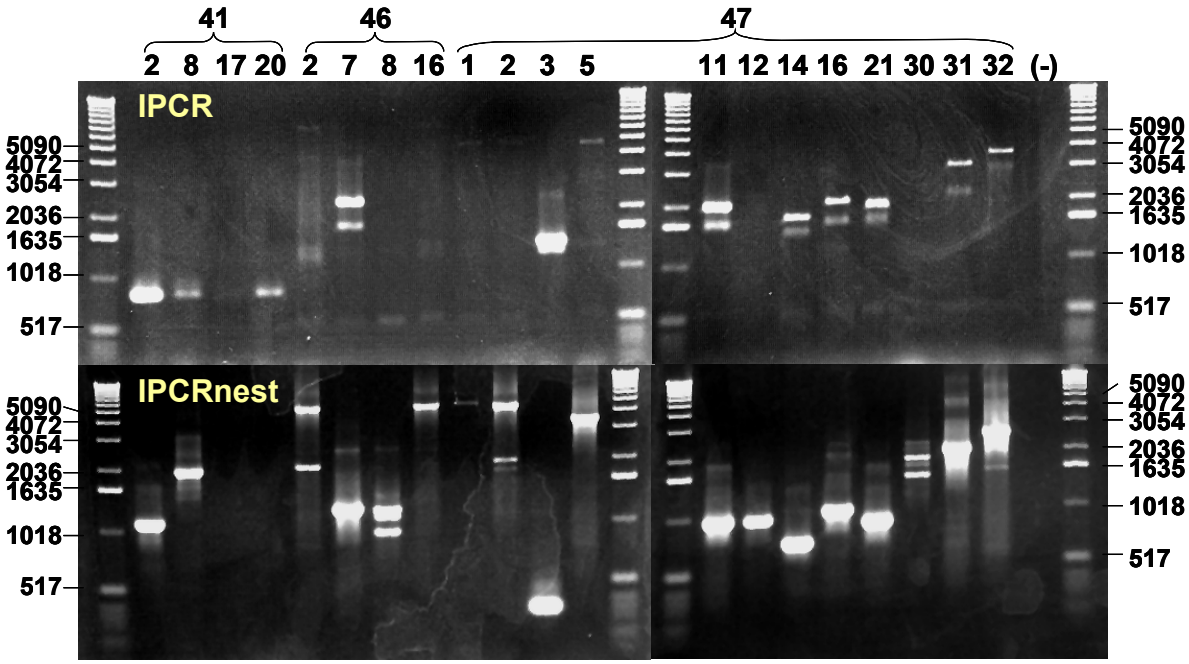


Figure 3-24 IPCR and subsequent nested IPCR. The products from IPCR (TSRIF/LAIRT) and nested IPCR (SELLA/GFPrevinv) with genomic DNA from all construct strains as templates were separated in a 0.8% agarose gel. The different constructs are indicated by their previously used numbering. A negative control reaction without template DNA is shown (-). The size of each marker fragment of the DNA size marker was as indicated.

Since all of the constructs with the *gfp* variant designated 41 showed a signal at 0.8 kb, it was assumed that this signal was not specific.

Although not all bands obtained with the nested IPCR could be matched with a corresponding signal in the first IPCR that was longer by 1.1 kb all good signals from the nested IPCR, were excised from the gel and submitted to sequencing (see 2.7). SELLA and GFPrevinv were used as sequence primers in two separate amplification reactions for each excised band.

Table 3-4 Amplicon Lengths with SELLA & GFPrevinv and TSRIF & LAIRT. The table shows the sizes of the bands that were visualised after IPCR and nested IPCR in the agarose gel electrophoresis. Numbers in parentheses are weak bands. Numbers in bold print are bands that were excised and sequenced.

Construct strain *Ps. putida* KT2440::mergfp	IPCR- Amplicon Length (TSRIF & LAIRT) [kb]	Nested IPCR- Amplicon Length (SELLA & GFPrevinv) [kb]	Amplicon Length Difference [kb]
41-2	0.8	**1.1** (2.2)	[1]
41-8	0.8	**1.9**	[1]
41-17	(0.8)	-	-
41-20	0.8	(**0.7**)	ns
46-2	1.4 (4.0; 7.0)	**5.0** (**2.0**; 0.5)	[1]
46-7	2.2 (4; 1.7)	1.3 (2.4)	0.9
46-8	(3.0; 1.4)	**0.9**; **1.3** (2.5)	ns
46-16	(7.0; 2.0; 1.8; 1.3; 0.5)	**4.8** (2.3)	2.2
47-1	5.0 (1.4; 0.5)	**5.0**	ns
47-2	5.0 (1.4; 0.5)	**4.8; 2.0** (1.8)	0.2; 3.0
47-3	1.2; 1.3 (2.3)	**0.3**	0.9; 1.0
47-5	4.7 (1.3; 0.5)	**3.5**	1.2
47-11	2.0;1.6 (2.5; 0.5)	**1.0** (1.8)	1.0; 0.6
47-12	(0.5)	**1.0**	[1]
47-14	1.6 (1.5;0.5)	**0.6**	1.0
47-16	2.1; 1.6 (0.5)	**1.1**	1.0; 0.6
47-21	2.0 (1.6; 0.5)	**0.9**	1.1
47-30	-	**1.9**; **1.5** (1.8)	[1]
47-31	3.1 (2.2)	**2.0**	1.1
47-32	3.8 (3.0)	**2.7**	1.1

[1] nested PCR product exceeded original PCR in size

[2] ns = not specified

3.3.1.3 Genetic Characterization of the Construct

For 15 out of the 20 constructs the genomic DNA sequence immediately next to the insertion could be revealed by IPCR and subsequent sequencing (Figure 3-25 & Figure 3-26, Table 3-5). The sequence could not be elucidated for *Ps. putida* KT2440::mergfp41-7, 41-20, 46-16, 47-1 and 47-3 owing either to a lack of or insufficient IPCR product resulting in no or bad sequence data. Sequences were compared with the corresponding chromatogram (data not shown) and bases corrected if necessary.

Genetic differences of the new mercury reducing, fluorescent constructs could be observed at several levels (Figure 3-25 & Figure 3-26): (1) The genotypes differed by definition by the

respective integration sites of the *mergfp* cassette in the genome of the recipient. (2) As has been described earlier, three groups could further be categorized by the *gfp* version used in the construction (derived from pBJA41, pBJA46 or pBJA47, with different half lives of the protein, Table 2-1). (3) Furthermore, as a consequence of the blunt end ligation of the *mer-gfp* cassette into the transposon vector, the *gfp* was flanked by the I-end in the constructs from the 41 and 47 series, while in the constructs from the 46 series, *gfp* was flanked by the O-end. This had no consequence for the transposition of the *mergfp* cassette or later gene expression. Nevertheless, the fact had to be acknowledged for the analysis of the genetic data.

An alignment of the *mergfp* cassette - genomic DNA transitional region of the constructs was performed using ClustalX (1.64b; Protein Weight Matrix: BLOSUM series, Gap Opening 15, Gap Extension 6.66) and editing was carried out with GeneDoc-Multiple Sequence Alignment Editor & Shading Utility, version 2.6.002. With this alignment the genetic differences among the constructs could clearly be discerned (Figure 3-25).

The insertion site sequences of 47-11 and 47-12 proved identical. In their mercury reduction kinetics and fluorescence intensity, however, the two constructs could clearly be differentiated (3.3.4), it is therefore highly unlikely that the two strains would have been identical. The clone 47-12 did not give a signal in the IPCR, but only in the nested PCR, so that it may be that the latter signal was an artefact probably caused by contamination.

Another noteworthy observation in Figure 3-25 were the purple or turquoise patches at the beginning of the genomic DNA. The first base column after the O-end shows 80% identity among the constructs. The 4^{th}, the 6^{th}, the 9^{th} and the 11^{th} base column after the O-end still show 60% identity, while after that base identity remains below 60%. The accumulation of identical base pairs seemed to be locally confined to the 11 bases downstream the integration, suggesting a site selection for the Tn5 transposition (also see 4.2.2).

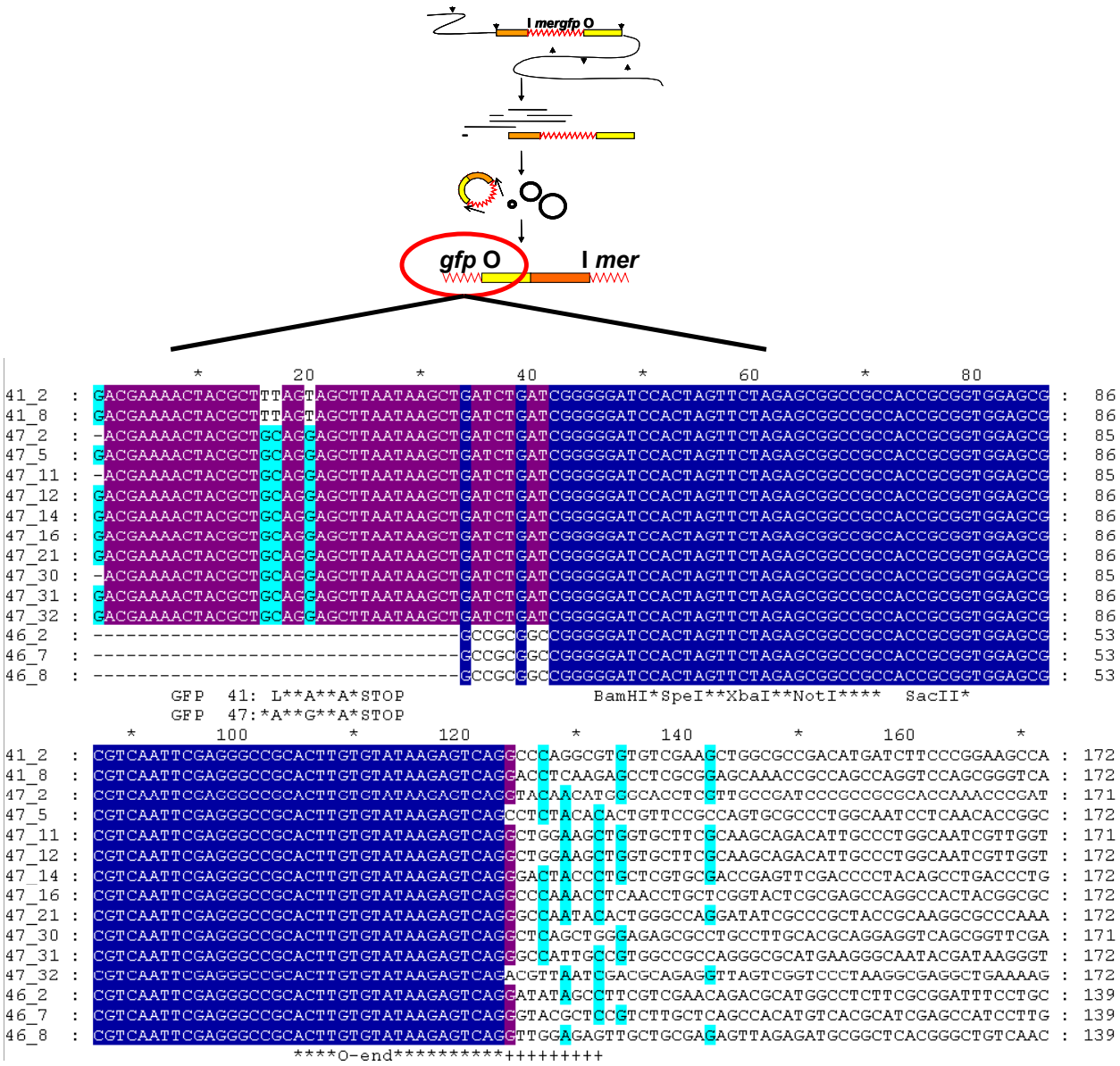


Figure 3-25 Genetic Differentiation of the Constructs: Genomic *Ps. putida* DNA bordering the O-end of the *mer-gfp* insertion. Sequence primer was GFPrev (41 series & 47 series) or SELLA (46 series). The alignment shows the 3' end of *gfp* (41 and 47 series), the partial multiple cloning site and the O-end of pBSL299ΔSm. Note that in the constructs of the 46 series the *mer* operon, and not the GFP gene, is flanked by the O-end (not shown). The two GFP versions shown differ in their last three amino acids. Shading was chosen as follows: Blue indicated complete identity, purple was used if 80% and turquoise if 60% of the bases in one column were identical. White indicated no similarity. Nine bases of genomic *Ps. putida* KT2440 DNA were duplicated at the insertion site during the integration and are indicated with (+). For further information see text.

By enzyme restriction digest of the construct DNA with *Kpn*I, the I-end and its immediately flanking genomic region were cut off due to a *Kpn*I recognition site in the multiple cloning site of pBSL299ΔSm (Figure 3-17). Hence, the *Kpn*I fragment holding the *mer-gfp* cassette comprised at one end partial multiple cloning site and genomic DNA at the other end. When

self-ligated the genomic DNA immediately flanking the *Kpn*I site of the *mer-gfp* cassette therefore presented the genomic DNA further upstream the O-end and not the downstream DNA immediately flanking the I-end (Figure 3-26). However, the obtained genomic sequence bordering the *Kpn*I site proved extremely valuable for verifying the integration site of a particular construct strain. The insertion sequence was identified by comparing the genomic sequence flanking the O-end with the *Ps. putida* KT2440 genome database (see Figure 3-28). Only if the genomic sequence bordering the *Kpn*I site could be found further upstream of the identified integration site in the *Ps. putida* KT2440 genome the place of insertion could be confirmed.

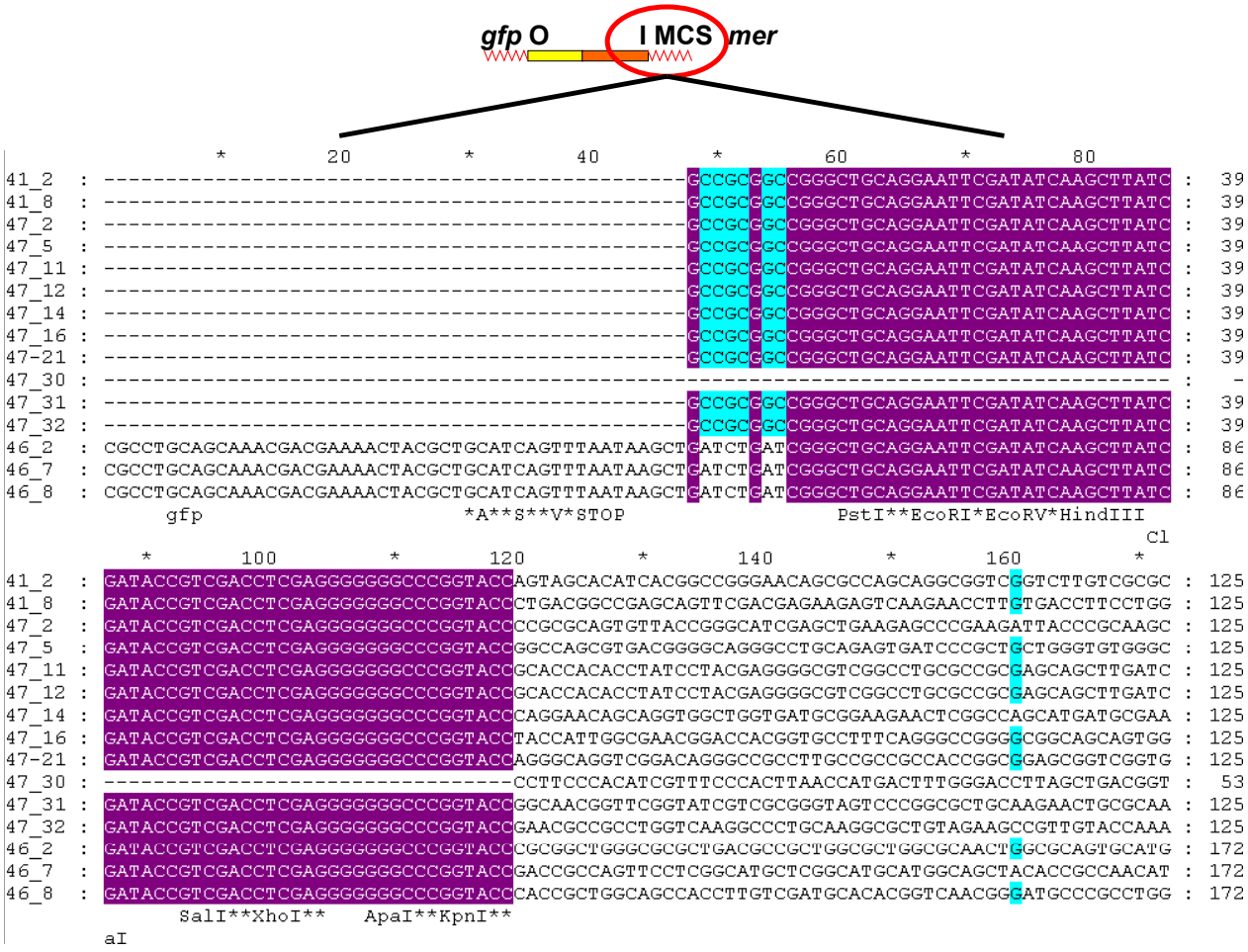


Figure 3-26 Genomic *Ps. putida* DNA bordering the *Kpn*I site of the self-ligated fragment. Sequence primer was SELLA (41 series & 47 series) or GFPrevinv (46 series). The alignment shows the DNA upstream the 5' end of the *mer* operon (41 series and 47 series only) and the partial multiple cloning site of the Tn5-minitransposon. Note that in the constructs of the 46 series *gfp*, and not the *mer* operon, is flanked by the I-end (not shown). Shading was chosen as follows: Purple indicated 80% and turquoise 60% identity of the bases in one column. White indicated no similarity. Genomic *Ps. putida* KT2440 DNA begins at the transition of purple to white. Note that the *Kpn*I digest produced fragments with genomic DNA at one end and the pBSL299ΔSm multi cloning site at the other end. Hence, after self-ligation of that fragment, the genomic DNA upstream the *mer* genes in the construct was found bordering the cassette at the *Kpn*I site. MCS=multiple cloning site.

3.3.2 Localization of the Integration Sites

To localize the insertion sequence within the *Ps. putida* KT2442::mer73 genome, the genomic sequence was initially compared with the contig database available from the *Ps. putida* KT2440 Genome Project (The Institute for Genomic Research - TIGR/ German Consortium, Prof. Dr. KN Timmis, "http://www.tigr.org"). *Ps. putida* KT2440 and *Ps. putida* KT2442 (who was the parent for the construction of the GEM) are genetically identical except for a single base mutation rendering the latter resistant to rifampicin. The GEM sequence flanking the *mer* operon integration could be found within contig #10458 (March 2000), and with this information could be located within 50 – 60 kb from the *Swa*I restriction site separating the fragments SwD and SwB (German Consortium, Prof. Dr. B. Tümmler, Figure 3-27).
With the combined physical and genetic map of the *Ps. putida* KT2440 genome of Ramos-Díaz & Ramos (1998) the insertion site of the *mer* operon in *Ps. putida* KT2442::mer73 could be located in genomic neighbourhood to *rpo*S (RpoS- σ factor) on the SwB fragment.

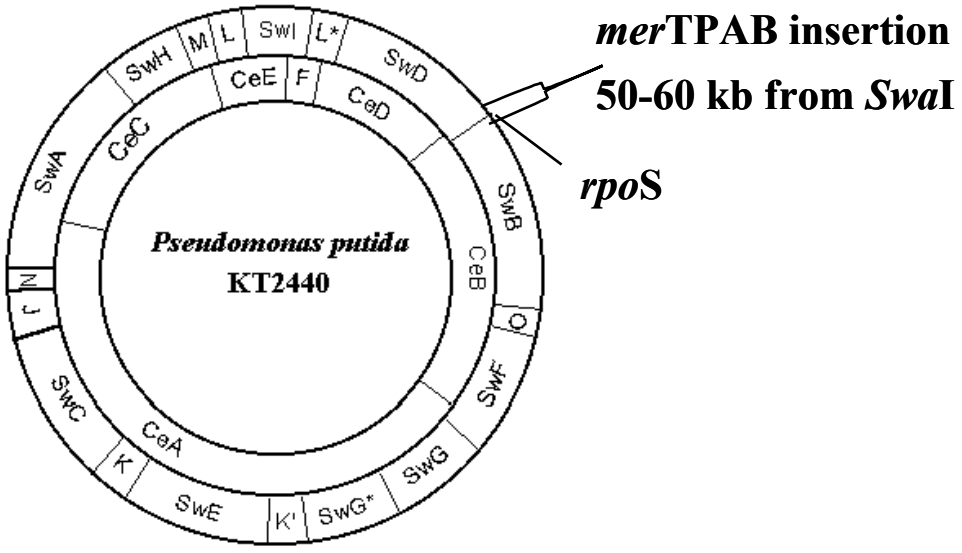


Figure 3-27: *Pseudomonas putida* KT2440 genomic map and insertion of *mer*TPAB in *Ps. putida* KT2442::mer73. The circular chromosome is represented as a series of fragments of the 6 MB *Ps. putida* KT2440 genome when cut with the endonucleases *Swa*I (Sw, outer circle) and I-*Ceu*I (Ce, inner circle) (Ramos-Díaz & Ramos 1998). The site of insertion of the *mer* operon into the *Ps. putida* genome is indicated as well as the location of the *rpo*S gene on the SwB fragment. Figure modified, original figure kindly provided by Christian Weinel (MH Hannover).

3.3.3 Identification of the mer Operon Insertion Site with the Annotated Ps. putida KT2440 Genome

Only recently, the *Ps. putida* KT2440 genome was published and the annotated sequence submitted to NCBI where it can be obtained with the accession number NC_002947 (http://www.ncbi.nlm.nih.gov:80/cgi-bin/Entrez/framik?db=genome&gi=266, Peterson *et al*. 2001, Nelson *et al*. 2002,). With this information the insertions could be further identified in terms of affected gene functions for *Ps. putida* KT2442::mer73 and for the new constructs.

The obtained sequences were submitted to the *Ps. putida* KT2440 genomic DNA database using BlastN (Figure 3-28). This search rendered an alignment of the submitted sequence with the matching *Ps. putida* KT2440 genomic region. Furthermore the numbers of the corresponding basepairs were gained according to the database numbering which were crucial for the identification of the annotated function to the DNA sequence received in this search.

For the new constructs only the region immediately adjacent to the O-end represented the integration site, because of a *Kpn*I cut site immediately after the *gfp*.

```
Query: 1       gcccaggcgtgtgtcgaagctggcgccgacatgatcttcccggaagccatcaccgaactg 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2662416 gcccaggcgtgtgtcgaagctggcgccgacatgatcttcccggaagccatcaccgaactg 2662475

Query: 61      cagatgtacaagactttcgctgatcgggtgaaggcaccgatcctggccaacatcaccgag 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2662476 cagatgtacaagactttcgctgatcgggtgaaggcaccgatcctggccaacatcaccgag 2662535

Query: 121     ttcggtgccacgccgctgtacacaaccgaagagctggcctcggtcgacgtgtcgttggtg 180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2662536 ttcggtgccacgccgctgtacacaaccgaagagctggcctcggtcgacgtgtcgttggtg 2662595

Query: 181     ctgtacccgctgtcggcgttccgcgccatgaacaaagcagccg 223
               |||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2662596 ctgtacccgctgtcggcgttccgcgccatgaacaaagcagccg 2662638
```

Figure 3-28 Finding the Integration Site. By BlastN alignment of the construct sequence obtained from the *Kpn*I IPCR signal ("Query", here shown for genomic DNA adjacent to the O-end in *Ps. putida* KT2440::mergfp41-2) with the *Ps. putida* KT2440 genome (Subject) the insertion site could be localized in terms of base number (bold print). In *Ps. putida* KT2440::mergfp41-2 bases 2662416 to 2662424 were duplicated as the *mergfp* cassette was integrated into the genome (bold print). See text for further information.

For *Ps. putida* KT2442::mer73 and for the 15 constructs for which good sequences had been obtained the insertion sites could be described in terms of gene environment and gene disruption (Table 3-5). Although the *Ps. putida* KT2440 genome has now been fully sequenced many gene functions remain unknown. Open reading frames that could not be annotated have been designated hypothetical protein or conserved hypothetical protein on the basis of sequence similarities with other proteins found in databases. It would be

presumptuous to infer that these genes were of no great significance to the bacterium, however, their relevance is not presently known. Among the 15 constructs more than one third of the identified integrations were within hypothetical proteins that could not further be identified (*Ps. putida* KT2440::mergfp46-2, 47-2, 47-11, 47-12, 47-21, 47-31). Disruption of the gene in these cases did not exterminate survival of the cell nor did it deteriorate growth, at least not in M9 medium with benzoate (Figure 3-29).

In some cases, the insertion occurred within or between genes that were assigned to a group of proteins but were no further characterized (putative carboxyvinyl carboxyphosphonate phosphoryl synthase of *Ps. putida* KT2440::mergfp41-2, putative lipoprotein of *Ps. putida* KT2440::mergfp41-8, *soh*B protein of the U7 peptidase family and phosphoglycerate mutase family protein of *Ps. putida* KT2440::mergfp46-8, decarboxylase family protein of *Ps. putida* KT2440::mergfp47-5).

Other genes with the same annotated functions were found within the genome for putative carboxyvinyl carboxyphosphonate phosphoryl synthase of *Ps. putida* KT2440::mergfp41-2, putative lipoprotein of *Ps. putida* KT2440::mergfp41-8, phosphoglycerate mutase family protein of *Ps. putida* KT2440::mergfp46-8, 3-dehydroquinate dehydratase (*aro*Q) of *Ps. putida* KT2440::mergfp47-16, tRNA-Ala-6 of *Ps. putida* KT2440::mergfp47-30 and Pp23SF-rRNA of *Ps. putida* KT2440::mergfp47-32. These multiple present genes could have prevented the insertion from being lethal by surrogating the function of the disturbed gene.

In two cases the insertion occurred between two genes (between the small and large acetolactate synthase subunits of *Ps. putida* KT2440::mergfp46-7 and in *Ps. putida* KT2440::mergfp46-8 between a phosphoglycerate mutase family protein and a *soh*B protein, peptidase U7 family, that could not further be characterized). In *Ps. putida* KT2440::mergfp46-7, however, the cassette insertion cut off the stop codon of the large subunit, so that translation would have continued into the sequence of the cassette, possibly compromising the function of the enzyme. Although other acetolactate synthases were present in the genome, this particular biosynthetic type (*ilv*N, *ilv*B) that is involved in amino acid synthesis was only present once. In *Ps. putida* KT2440::mergfp46-8 the insertion took place between two genes that were regulated in opposite directions, on the sense and the antisense strand. The question that needed answering was if although the genes themselves were not affected by the insertion, their regulatory DNA sequences might have been affected. Promoter regions were searched for with the Baylor College of Medicine (BCM) Search Launcher (http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html). The insertion was found between the regulatory region of the phosphoglycerate mutase family protein and the gene. In

fact two promoter regions for the gene could be identified, which might be responsible for the good performance and fluorescence of the strain. The enhanced distance to the promoter could, however, have resulted in a deteriorated transcription of the phosphoglycerate mutase. Nevertheless, other similar genes were present that could have taken over the function. The promoter for the *soh*B family protein could not be found within 1000 bp upstream the start codon. The gene might have been transcribed from a promoter that was further away or the ORF that was found and annotated was not a functional gene *in vivo*. This finding subscribes the need for expressional studies beyond mere genetic studies.

An exception among the insertions was the γδ electron transferring subunit of the NADH dehydrogenase I, also called NADH:ubiquinone oxidoreductase (*nuo*CD) or complex I which was only present once in the genome. The NADH dehydrogenase I consists of 13 subunits and is the first component of the respiratory chain with the lowest redox potential of all components. It is hence an essential enzyme for respiration and thus for growth of the aerobic *Ps. putida*.

In *Ps. putida* KT2442::mer73 the *mer* operon was inserted into the alanyl-tRNA-synthase sequence about 130 bp towards the 3' end of the ORF. The next ORF downstream was a putative major-facilitator-superfamily (MFS)-transporter. Although the insertion did occur within a coding sequence, apparently it did not destroy gene function of *Ps. putida* KT2442::mer73. *Ps. putida* KT2440 harbours only one gene coding for alanyl-tRNA-synthase which is essential in protein biosynthesis and the GEM would not have survived if the insertion would have been lethal (see 4.2.1 for further discussion).

Table 3-5 Integration sites of the *mergfp* cassette in the constructs and of the *mer* operon in *Ps. putida* KT2442::mer73.

GFP-version (last 3 amino acids, half life)[1)]	Construct Strain [*Ps. putida* KT2440::mergfp]	Orientation-localization	Disrupted gene
41 LAA $t_{1/2}$= 2½ min	**41-2**	O-end-*gfp-mer*-I-end,	bp 2662416-24 (PP2334) carboxyvinyl-carboxyphosphonate phosphorylmutase, putative
	41-8		bp 1508422-30 (PP1322) lipoprotein, putative
	41-17		ND
	41-20		ND
46 ASV $t_{1/2}$= 6 min	**46-2**	I-end-*gfp-mer*-O-end	bp 5189985-93 (PP4570) conserved hypothetical protein
	46-7		bp 5317650-58 (between PP4679 and PP4680) acetolactate synthase, small subunit and large subunit (*ilv*N,*ilv*B)
	46-8		bp 4428945-53 (between PP3922 and PP3923) *soh*B protein- peptidase U7 family, phosphoglycerate mutase family protein
	46-16		ND
47 AGA $T_{1/2}$= >30 min	**47-1**	O-end-*gfp-mer*-I-end	ND
	47-2		bp 4328586-94 (PP3798) conserved hypothetical protein
	47-3		ND
	47-5		bp 4159361-69 (PP3662) decarboxylase family protein
	47-11[2)]		bp 2014141-48 (PP1794) hypothetical protein
	47-12[2)]		bp 2014141-48 (PP1794) hypothetical protein
	47-14		bp 4657856-64 (PP4121) γδ NADH-dehydrogenase I subunit (*nuo*CD)
	47-16		bp 650676-84 (PP0560) 3-dehydroquinate dehydratase, type II (*aro*Q)
	47-21		bp 3486177-85 (PP3092) conserved hypothetical protein
	47-30		bp 699539-47 (tRNA-Ala-6) tRNA
	47-31		bp 2797630-38 (PP2450) conserved hypothetical protein
	47-32		bp2552025-33 (PP23SF) rRNA
--	***Ps. putida* KT2442::mer73**	O-end-*mer*-I-end	bp1270955-63 (PP1111) synthetase, putative

ND - not determined

[1)]These half lives were determined for *E. coli* (personal communication with M. Strätz and B. Andersen), see section 3.2.2.

[2)]Clones 47-11 and 47-12 were identical (compare alignment)

3.3.4 Growth in M9 Minimal Medium (+Benzoate)

By observing the growth of the bacterial construct strains, a first evaluation of their physiological intactness despite the insertion could be achieved. The new *Ps. putida* KT2440::mergfp constructs, the parent strain *Ps. putida* KT2440 and the mercury reducing constructs *Ps. putida* KT2442::mer73 and *Ps. putida* KT2442::mer::gfp11 (Suarez *et al*. 1997) were grown with M9 minimal medium and benzoate as sole carbon source (Figure 3-29). *Ps. putida* KT2442::mer::gfp11 is a mercury resistant, fluorescing strain that contains a stable GFP version, which is, however, not linked with the mercury resistance. All strains grew to similar maximum optical densities. However, *Ps. putida* KT2442::mer73, *Ps. putida* KT2440::mergfp46-2 and 46-7, *Ps. putida* KT2442::mer::gfp11 grew with a delayed lag phase while *Ps. putida* KT2440::mergfp46-8 decelerated its growth in mid-log phase. Nevertheless, this growth experiment shows preliminary results that need to be validated.

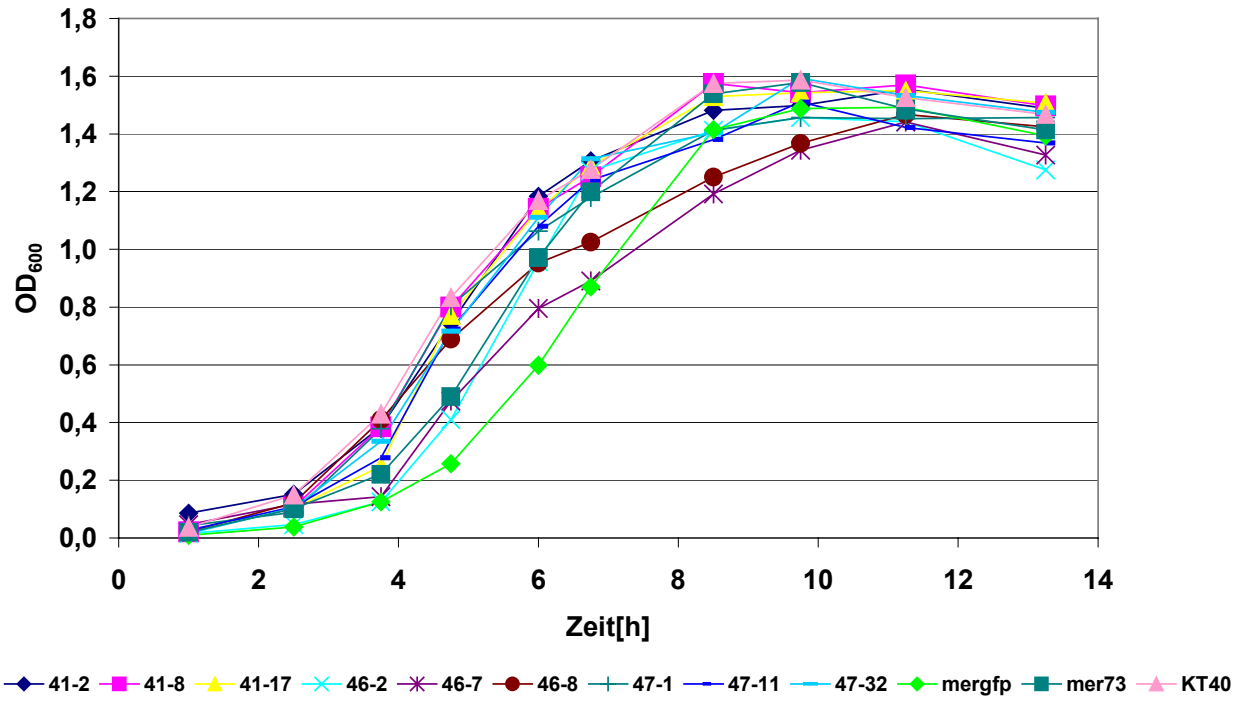


Figure 3-29 Growth of constructs in M9 minimal medium with benzoate as sole carbon source. Growth curves are shown for nine of the constructs that had been selected for their mercury resistance. The constructs are specified by their numbers. The parent strain *Ps. putida* KT2440 (KT40) was included into the growth experiment as well as the mercury resistant strain *Ps. putida* KT2442::mer73 (mer73) and *Ps. putida* KT2442::mer::gfp11 (mergfp). The latter possesses green fluorescent protein in addition to the mercury resistance.

3.3.5 Expression of Mercury Resistance and GFP

3.3.5.1 Fluorescence During Colony Growth

Although the new *Pseudomonas* constructs were selected for their capability to transform mercury, an important feature was their fluorescence, enabling easy detection of the constructs in complex bacterial consortia as well as quantification of the mercury resistance activity via fluorescence intensity.

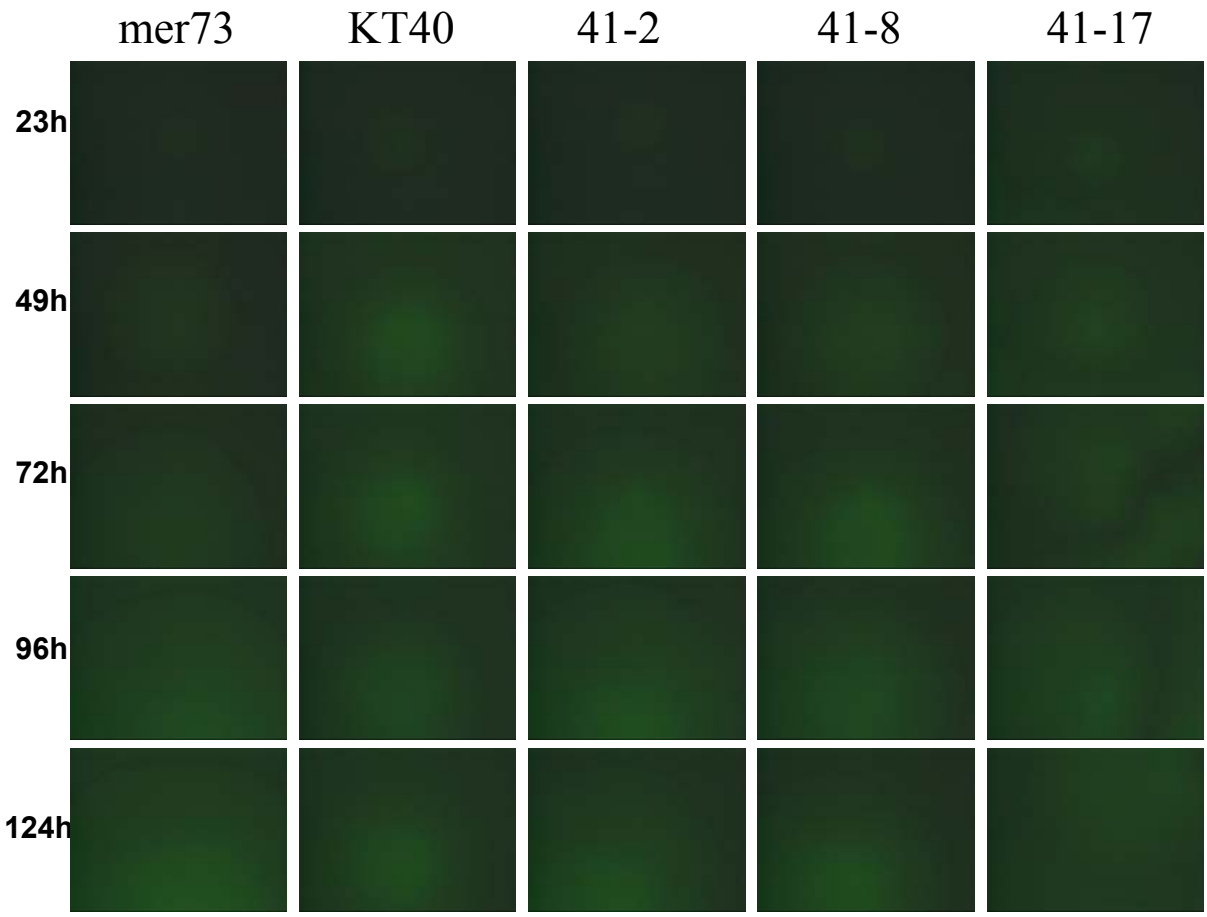


Figure 3-30 Green Fluorescence during colony growth in the 41 series. Fluorescence is shown for single colonies of the *Ps. putida* KT2440::mergfp constructs carrying the most unstable of the three GFP versions (Andersen *et al*. 1998). For comparison, *Ps. putida* KT2442::mer73 ("mer73") and the parent strain *Ps. putida* KT2440 ("KT40") are also shown. The magnification was ×25, colonies were observed with a FITC filter set (see 2.9.1).

In order to determine which construct strains showed highest fluorescence, growing colonies were observed over a period of 170 h with a fluorescence microscope, a 2.5× objective and FITC Filter Set III (see also 3.3.5.1). Figures 3-27 to 3-29 show the fluorescence during colony growth of three construct strains from each series (41, 46, 47) with the strongest

fluorescence as determined by eye. After 124 h the fluorescence intensity and size of the colonies did not change, therefore later pictures have been omitted in the figures.
The most striking observation was that the constructs with the most unstable GFP-version (the 41-series) showed only very low fluorescence that was not appreciably higher than the auto-fluorescence of the *Pseudomonas* parent strain (compare *Ps. putida* KT2440; Figure 3-30).

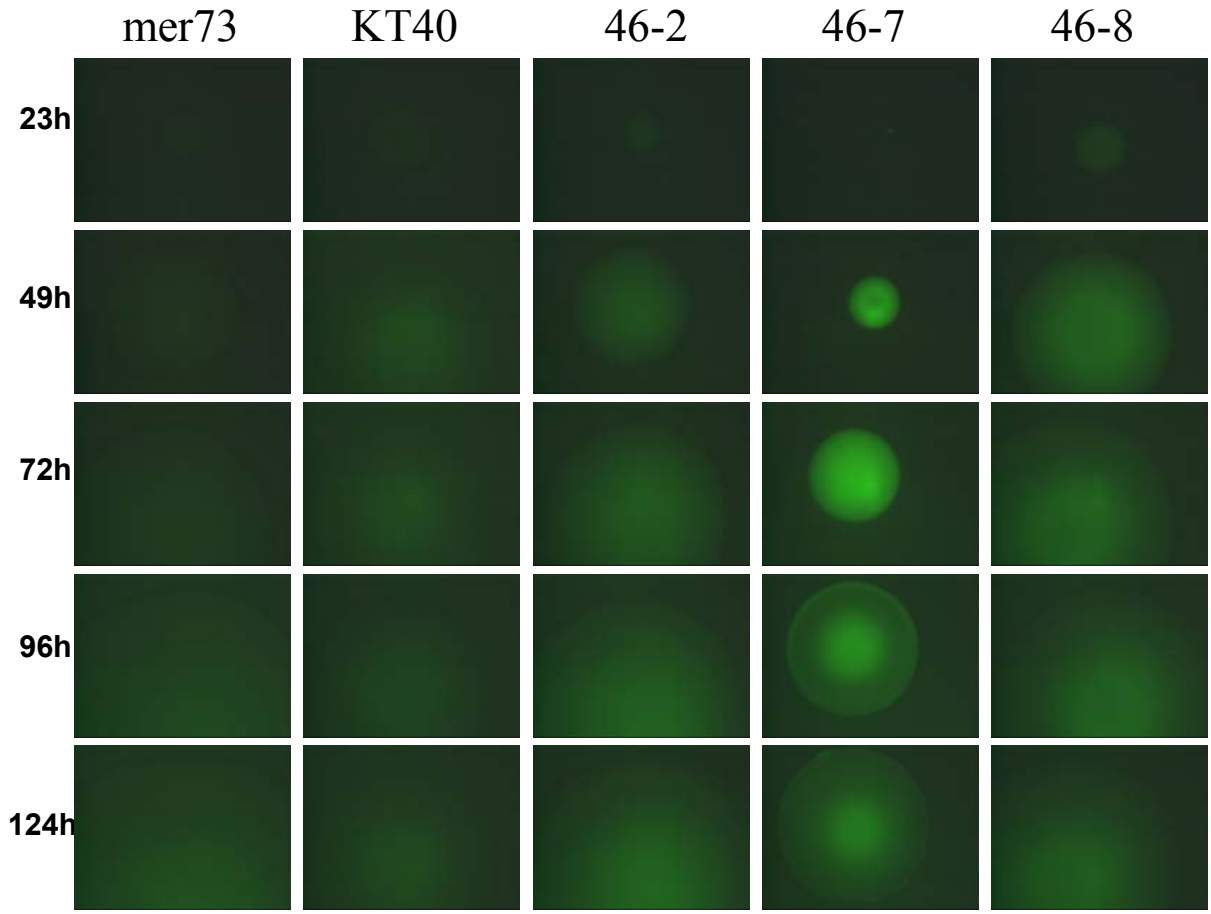


Figure 3-31 Green Fluorescence during colony growth in the 46 series. Fluorescence is shown for single colonies of *Ps. putida* KT2440::mergfp constructs carrying a moderately stable GFP versions with a half time of 6 min in *E. coli* (Andersen *et al*. 1998). For comparison, *Ps. putida* KT2442::mer73 ("mer73") and the parent strain *Ps. putida* KT2440 ("KT40") are also shown. The magnification was ×25. Colonies were observed with a FITC filter set (see 2.9.1).

Most of the constructs with the GFP-version that was more stable (the 46-series) displayed a fluorescence intensity that was clearly higher than the *Pseudomonas* auto-fluorescence (Figure 3-31), while many constructs with the most stable of the three GFP-version (the 47-series) were by bright fluorescent, comparable with *Ps. putida* KT2442::mer::gfp11 (Figure 3-32) that carries a stable GFP version in its chromosome (Suarez *et al*. 1997). Both the 41-series and the 46-series were under-represented (4 construct strains each) in comparison to the

47-series (12 construct strains), thus this phenomenon may be attributed to statistical error. However, a correlation of increasing fluorescence intensity with an increase in GFP stability could clearly be observed. Besides the varying GFP fluorescence intensities among the different construct strains, changes in intensity could also be observed within a single colony during its growth. In *Ps. putida* KT2440::mergfp46-7 this could be easily observed (Figure 3-31). In the young colony (23 h) fluorescence was uniformly distributed but soon (49 h) the core became darker in comparison to a ring of medial distance from the centre. However, the edges of the colony remained dark. At later colony growth stages (72 h) the whole colony fluoresced uniformly, later (96 h) fluorescence ceased except for the edges and the core region, fluorescing fading at the borders (124 h).The construct strains of the 47 series also displayed spatial differences of fluorescence during colony growth (Figure 3-32). While not very pronounced in *Ps. putida* KT2440::mergfp47-11 who fluoresced homogeneously without the formation of fluorescing rings, *Ps. putida* KT2440::mergfp47-1 and *Ps. putida* KT2440::mergfp47-32 did show spatial variation of fluorescence within the colonies. *Ps. putida* KT2440::mergfp47-1 fluoresced uniformly from the beginning (23 h) but with a darker core. The colony then developed bright fluorescing borders and a broad ring of high fluorescence around the immediate centre (49 h). Fluorescence ceased first at the edges (72 h) and then more and more around the centre (96 h and 124 h). *Ps. putida* KT2440::mergfp47-32 showed the brightest fluorescence of the construct strains. The strain still fluoresced brightly after 170 h (data not shown, however, intensity was comparable with that at 124 h). At 23 h a slightly darker core region could be detected and the formation of fluorescent rings at 49 h. After that fluorescence faded slightly, however several fluorescent rings could be detected. Fluorescence ceased first at the borders while remaining bright towards the centre (72 h, 96 h, 124 h).

Moreover, while some colonies grew in close vicinity to neighbouring colonies, others had more space on the agar plate leading to a greater relative resource of nutrients. Therefore, fluorescence patterns could also have depended upon nutrient availability and should be confirmed in additional experiments.

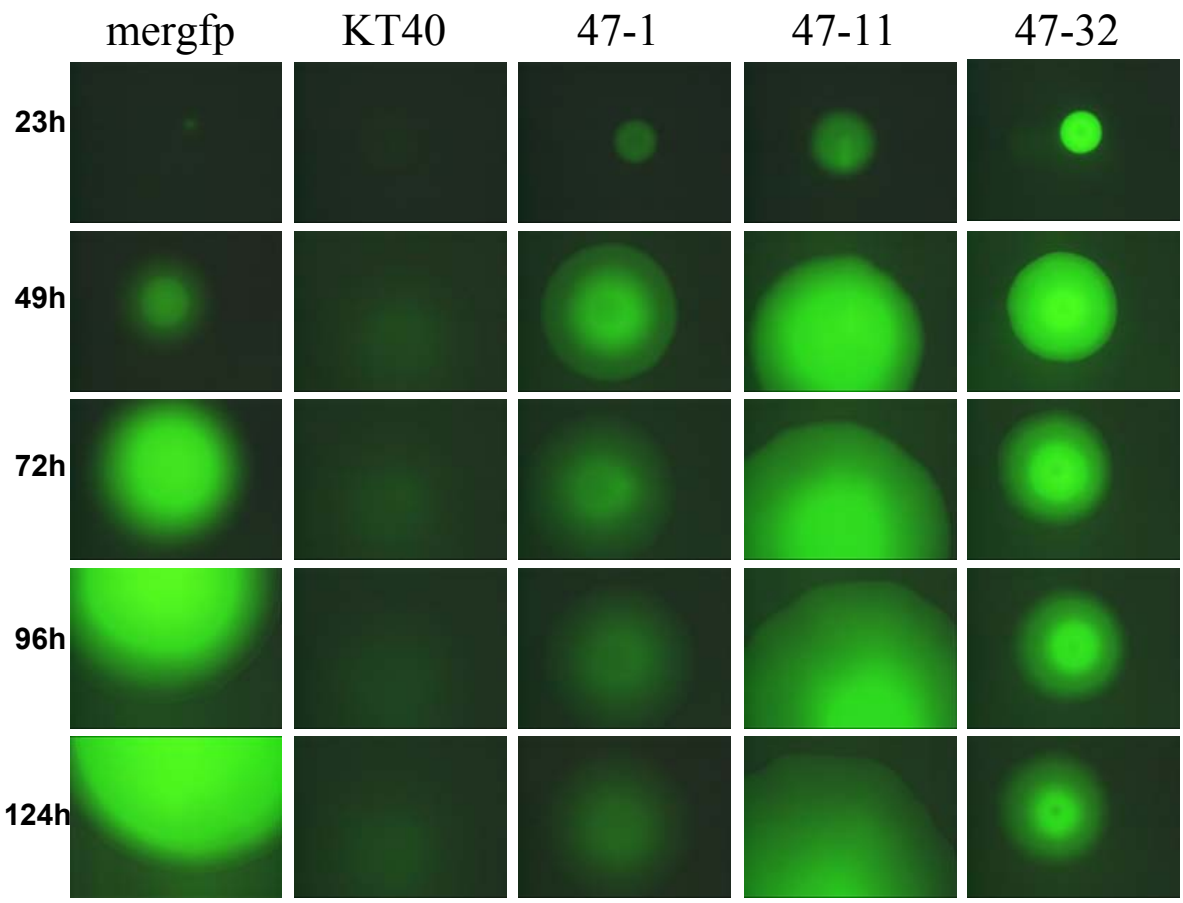


Figure 3-32 Green Fluorescence during colony growth in the 47 series. Fluorescence is shown for *Ps. putida* KT2440::mergfp constructs carrying the most stable of the three GFP versions with a half time of more than 30 min in *E. coli* (Andersen *et al*. 1998). For comparison, *Ps. putida* KT2442::mer::gfp ("mergfp") and the parent strain *Ps. putida* KT2440 ("KT40") are also shown. The magnification was ×25. The colonies were observed with a FITC filter set (see 2.9.1).

3.3.5.2 Correlating Mercury Resistance and Fluorescence

One of the prospects of creating transcriptional *mer-gfp* fusions was the possibility to monitor mercury-reduction by measuring GFP fluorescence. In order to investigate this possibility, samples were drawn at late logarithmic stage ($OD_{600\ nm}$ = 1.3) from M9 medium liquid cultures (+10 mM benzoate) and both, fluorescence and mercury reduction, were quantified. Mercury reduction is highest during logarithmic growth and decreases dramatically in stationary phase (personal communication with Wanda Fehr). Moreover, high cell densities were desired for a maximum of fluorescence facilitating quantification. Therefore, the late logarithmic growth phase was chosen for sampling.

3.3.5.2.1 Mercury Reduction Rates

From each construct series with a particular GFP variant three candidates with the best mercury resistance were chosen on the basis of the preliminary tests (compare sections 3.2.4, 3.3.4). For the determination of fluorescence intensity, 1 ml of the culture was measured in a fluorescence spectrophotometer (see section 2.9.2), with excitation at 475 nm and emission at 515 nm. For the determination of the mercury transformation rates, triplicate samples (1 ml) were injected into an Atomic Absorption Spectrometer (AAS; see section 2.10) and subjected to 3 ppm Hg^{2+} and the best performing strains later to 6 ppm Hg^{2+} ($HgCl_2$).

Four construct strains showed superior transformation rates with 3 ppm Hg^{2+}: *Ps. putida* KT2440::mergfp46-7, 47-1, 47-11 and 47-32 (Figure 3-33). If expressed as transformation rate per cell, however, *Ps. putida* KT2440::mergfp46-2 was superior to *Ps. putida* KT2440::mergfp47-1 which must be attributed to the extraordinary high cell density of *Ps. putida* KT2440::mergfp47-1 ($OD_{600\ nm}$ = 1.3) that was 5-10fold higher than that of the other construct strains (see Figure 3-34). Performance of *Ps. putida* KT2440::mergfp 47-1 thus seemed to be lower than *Ps. putida* KT2440::mergfp46-2. This observation could not be confirmed in the experiment with 6 ppm Hg^{2+}. Here, performance of *Ps. putida* KT2440::mergfp47-1 was second highest also if expressed as transformation rate per cell. The other five strains with high mercury transformation rates also possessed high mercury transformation per cell.

Since *Ps. putida* KT2440::mergfp46-7, 47-1, 47-11, 47-32, *Ps. putida* KT2442::mer73 and *Ps. putida* KT2440::mer::gfp11 showed enormously high mercury transformation rates with 3 ppm Hg^{2+} ($HgCl_2$), for these six strains, the mercury transformation rates were also determined with 6 ppm of Hg^{2+} (HgCl; Figure 3-35 & Figure 3-36) to further investigate their mercury reducing capacity: *Ps. putida* KT2440::mergfp46-7 had the lowest transformation rate of the construct strains, although comparable with *Ps. putida* KT2442::mer::gfp but lower than *Ps. putida* KT2442::mer73. All three strains of the 47 series showed higher mercury transformation rates than *Ps. putida* KT2442::mer73. *Ps. putida* KT2440::mergfp47-32 had the highest mercury transformation.

3.3.5.2.2 Survival During Mercury Transformation

Another approach to determine how well the construct strains could cope with mercury was to determine survival during the mercury transformation measurement when the strains were subjected to 3 or 6 ppm Hg^{2+} (Figure 3-34). Of *Ps. putida* KT2440::mergfp46-2, 46-7, 47-1, 47-11, 47-32, *Ps. putida* KT2442::mer73 and *Ps. putida* KT2442::mer::gfp11 between 1 and

10% survived the kinetic measurement, while a substantial number of cells died of all construct strains of the 41 series (lowest GFP stability), of *Ps. putida* KT2440::mergfp46-8 and of the parent strain *Ps. putida* KT2440. Of *Ps. putida* KT2440::mergfp41-8 and 46-8 no cell survived the measurement. When mercury transformation rates were determined with 6 ppm, *Ps. putida* KT2440::mergfp46-7 and *Ps. putida* KT2442::mer::gfp11 failed to survive. Of the strains tested, the constructs of the 47 series and *Ps. putida* KT2442::mer73 survived best during mercury transformation with 6 ppm Hg^{2+} (Figure 3-36).

3.3.5.2.3 Fluorescence and its Correlation with the Mercury Resistance

Differences in fluorescence could be observed between the GFP variants (Figure 3-33). While fluorescence of the most unstable (41) series was not above the non-fluorescent control strains (*Ps*. *putida* KT2440 and *Ps*. *putida* KT2442::mer73, which however showed some autofluorescence), in the construct strains of the 46 series (moderate stability) fluorescence was slightly higher than in the controls. In the constructs with the most stable GFP variant (47 series), fluorescence was notably higher than in the other strains. Since the more stable GFP versions retain more GFP molecules this result was not surprising. Although the mercury reducing, fluorescent *Ps. putida* KT2442::mer::gfp11 was included in the experiment as a control, intensity of fluorescence could not be directly compared the fluorescence of the construct strains since the *gfp* of *Ps. putida* KT2442::mer::gfp11 was not FACS optimised and fluoresced most intensely at 395 nm, and not at 488 nm.

In the 46 construct series mercury transformation rates from virtually none (*Ps. putida* KT2440::mergfp46-8, mercury transformation was not above control with *Ps. putida* KT2440) to high transformation rates (*Ps. putida* KT2440::mergfp46-7) were existent. This observed increase of mercury transformation with the increase in GFP stability, however, does not yet allow inferences about a correlation between mercury transformation and fluorescence. Due to the fact that strains with more stable GFP contain more molecules, crucially, fluorescence within one GFP construct series had to be regarded for which only few data were available.

Correlation coefficients were calculated with Equation 3. For the 41 and for the 46 series higher mercury transformation at 3 ppm mercury was correlated with higher (logarithmic) relative fluorescence (r_{41}=0.97, r_{46}=0.99), however, these calculations were based on three data points for each feature only. In the 47 construct series differences in mercury transformation between the strains could not be resolved well with 3 ppm. Therefore the measurements were repeated with 6 ppm Hg^{2+} ($HgCl_2$; Figure 3-35 and Figure 3-36). The

correlation coefficients between mercury transformation and (the logarithmic of) the relative fluorescence in the 47 series were $r_{47(3)}$=-0.61 for the measurements at 3 ppm mercury and $r_{47(6)}$=0.57. More data must be collected before a correlation of mercury transformation and fluorescence can be confirmed. While certainly more data from constructs with all different GFP variants would be informative, the preliminary experiments conducted here (Figure 3-33 to Figure 3-36) suggest that the constructs of the 47 series are most promising as they showed both, high mercury transformation and fluorescence. Alternatively, investigation of the 46 series could yield further insight as they demonstrated the greatest variation, both in mercury transformation and in fluorescence, among the strains.

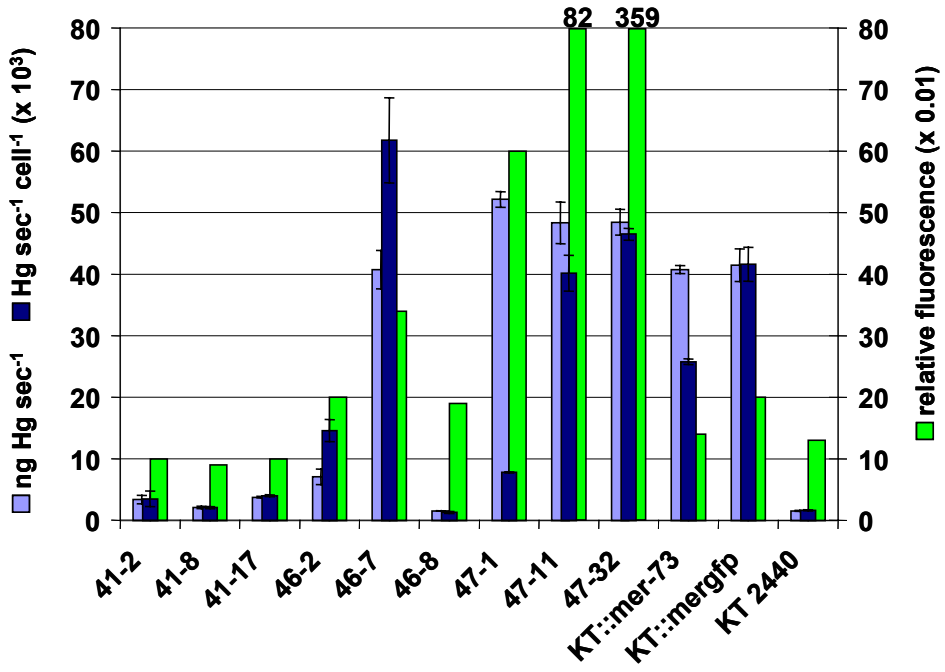


Figure 3-33 Mercury Transformation Rate with 3 ppm Hg^{2+} and Fluorescence of the *Ps. putida* KT2440::mergfp Construct Strains and controls. The relative fluorescence for *Ps. putida* KT2440::mergfp47-11 and 47-32 exceeded the scale and is indicated with numbers. Standard deviations from triplicates are shown for the mercury transformation rates.

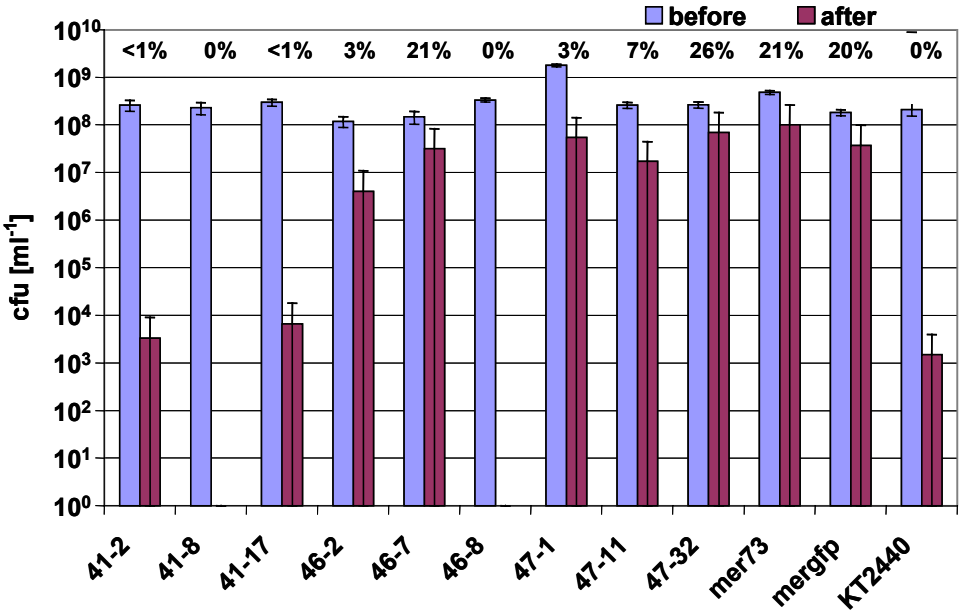


Figure 3-34 Survival During Transformation of 3 ppm Hg^{2+}. The figure shows the bacterial densities (and the standard deviation) in the vessel used to determine mercury transformation rates before and after the injections of 3 ppm Hg^{2+}. Survival of the cells is also expressed as percentage of surviving cells (for further information see text). No cells survived the mercury transformation measurement of *Ps. putida* KT2440::mergfp41-8 and 46-8.

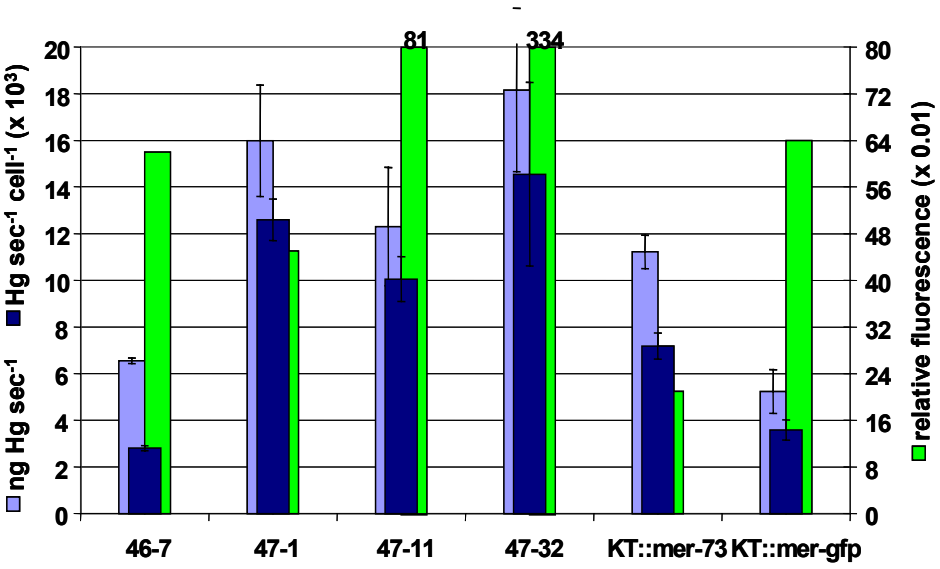


Figure 3-35 Transformation Rate of 6 ppm Hg^{2+} and Fluorescence of Selected Cells. For *Ps. putida* KT2440::mergfp47-11 and 47-32 relative fluorescence exceeded the scale and is indicated with a number. Standard deviations from triplicates are shown for the mercury transformation rates.

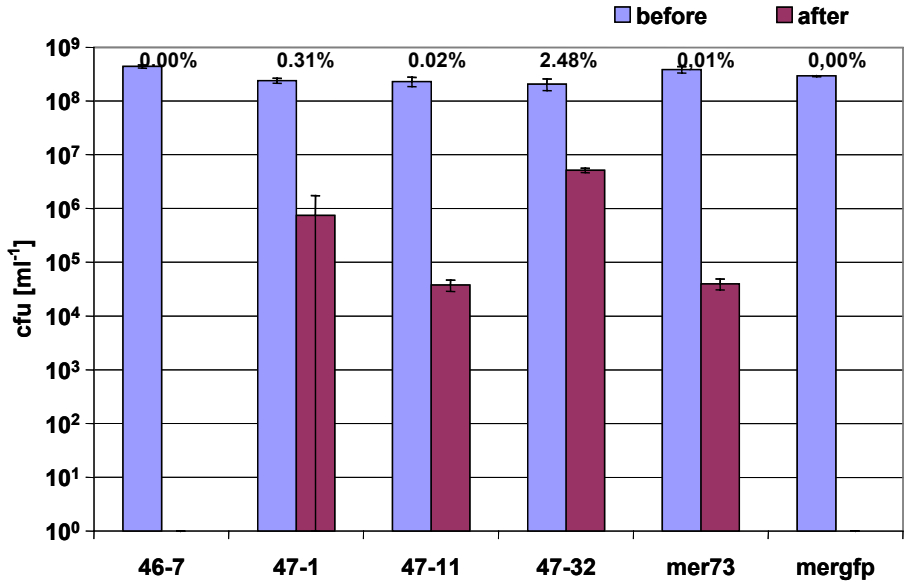


Figure 3-36 Survival During Transformation of 6 ppm Hg^{2+}. The figure shows the bacterial densities (and the standard deviation) in the vessel used to determine mercury transformation rates before and after the injections of 6 ppm Hg^{2+}. Survival is shown only for the best six construct strains and is also expressed as percentage of surviving cells (for further information see text). No cells survived the mercury transformation measurement of *Ps. putida* KT2440::mergfp46-7 and of the control strain *Ps. putida* KT2442::mer::gfp11.

4 Discussion

4.1 Microcosm Experiments - GEM Safety

4.1.1 Stream Microbiology

Streams and rivers are crucial mediators between terrestrial environments and the oceans. Consequently, most major cities developed near rivers that supplied water for drinking and irrigation (and fish, as a valuable source of nutrients) but furthermore rivers provided a means for the transport of goods and wastes. Rivers in nature run for several hundreds or thousands of Kilometres with tributaries or waste water influents and along its course a river may constantly face changing vegetation, geology or agricultural use (Allan 1995). Estimates of the amount of water discharged by rivers to the world's oceans range between 32000 and 37000 km^3yr^{-1}. Generally, flow rate can be considered the most important abiotic force in streams. This means that bacterial dissemination will be mainly controlled by current speed. As has been understood nowadays microbes play an important role in the cycling of elements and nutrients in the environment. Microbial carbon cycling and the degradation of toxins is particularly important in rivers that collect agricultural, municipal and industrial wastes. The benthos, i.e. all surfaces, including sediments, rocks, leaves, woods, etc., serves as matrix for microbial biofilms, a place of intricate microbial interactions, probably of frequent gene transfer, a place capable of somewhat protecting bacteria from predation or toxins, including antibiotics (Leff 1994). Brümmer *et al*. (2000) demonstrated the seasonal dynamics of river biofilm communities of Elbe and Spittelwasser, two German rivers which are polluted to different degrees, thereby showing the complexity of the river microbial communities and suggesting an influence of pollution on community composition.

Besides being the playground for microbial activity, benthic biofilms are also a great source of bacteria for the water column owing to abrasion by the current. The main characteristic of streams that distinguishes it from other aquatic systems is a longitudinal succession: everything is transported downstream. By so called spiralling of particles, however, everything (e.g. nutrients, bacteria, genes) settles at one point and stays for some time before released again, taken up by the current and transported further downstream.

4.1.2 Stream Microcosms

In this work, the fate of a mercury-reducing GEM and its influence on the indigenous microbial community was investigated. These experiments had to be carried out in an

artificial system –a microcosm- and since biofilms seemed to be a habitat with great microbial interaction it was assumed that an effect could most likely be seen in the near-bed environment, i.e. in the upper layer of the sediment that first came in contact with the GEM if it was introduced to the water stream above it. For the design of the experiments this also meant that lower stream velocities sufficed to represent the near-bed situation found in nature.

A new stream microcosm design requires thorough examination of its flow behaviour. In nature flow varies appreciably within the cross-section of a river. While common current velocities range between 0.01 and 1 m s^{-1} (Silvester & Sleigh 1985), speed decreases towards the bottom and the sides of the channel. In these regions, the boundary layer greatly protects the residing biota from being swept away by the current (Allan 1995). Flow patterns and velocity control the availability of nutrients and the forces applied on organisms living within the stream and at the water-sediment interface (Allan 1995, Silvester & Sleigh 1985, Vogel 1989). The surface velocity of the Elbe River at the time of sampling was 0.45 m sec^{-1}. Even with seasonally fluctuating discharge volumes this speed should not vary much as "[...] mean current speed at a particular station along a river only doubles for every tenfold increase in discharge." (Vogel 1989). The Elbe River surface water velocity was 136fold higher than the visually determined speed in the microcosm. However, velocity is reduced to near zero towards the river bed. As can be seen in Figure 3-2, flow very near the sediment layer was slow and the dye only reached down to the bottom after the dye front had nearly reached the second collimator. Therefore, near-bed velocity under natural river conditions was reflected well by the microcosm.

In the environment laminar flow, i.e. usually shallow, very slow moving water, is rarely found and turbulence mostly the rule. Near the sediment, turbulence mainly depends on the roughness if the surface. The Reynolds number describes the turbulence of a moving gas or liquid (see 3.1.2) and was used here for the purpose microcosm characterization. Flow in this microcosm was not highly turbulent, however, not laminar either. With 463 the Reynolds number indicated transitional flow, thus resembling real environmental conditions.

Experiments carried out in complex microcosms are associated with high experimental work loads and costs associated with construction and operation of the microcosms. By using (stream) microcosms that could be operated in a laboratory an attempt was made, to find a way between finding a precise answer to the scientific question posed, however not ignoring the (largely unknown) natural complexity of the system.

It is impossible to consider all possible parameters that would influence the survival and/or proliferation of the GEM in a river. However, the system for GEM safety studies does represent a river environment as realistically as possible.

Some loss of controllability in comparison to simple microcosms (see 3.1.1) is reconciled by the retention of at least some of the complexity of the real environment. Although others (e.g. Jungmann *et al*. 2001) have constructed similar indoor streams to investigate the ecology of benthic or lotic macroinvertebrate communities their microcosms were operated as closed-circuit systems or were filled with pebbles as sediment. The microcosms described here can be universally applied to investigate stream bacterial ecology (Leff 1994) and are novel in this application.

4.1.3 Survival of the GEM: Persistence and Dissemination

Survival of introduced bacteria depends on several biotic and abiotic factors (Smalla *et al.* 1989). Nutrients as well as predation (Jürgens *et al*. 1999) are major determinants. However, abiotic factors, such as temperature, pH, oxygen demand, water activity play additional roles in the adaptation process. It has also been speculated that viruses play an important role in the mortality of bacteria (Thingstad *et al*. 1993), however, evidence has been sparse. Nevertheless, "top-down" control as achieved by predators or viruses and "bottom-up" control e.g. through the limitation of nutrients are the major factors contributing to the abundance of bacteria. Liang *et al*. (1982) suggest "that some species persist in environments in which they are not indigenous because they tolerate abiotic stresses, do not lose viability readily when starved, and coexist with antagonists. The species that fails to survive need only be affected by one of these factors". So far, GEM survival in rivers has mainly been monitored by inoculation with large cell numbers of the strain at the beginning of the experiments and following the die-off (e.g. Iwasaki *et al*. 1993, Janakiraman & Leff 1999, Leff *et al*. 1997, Pechurkin *et al*. 1999). However, a feasible scenario is leakage of the GEM-containing system and subsequent escape of relatively small numbers of bacteria over a considerable time period. Despite rapid dilution of the GEM by the current, establishment of the strain would be conceivable due to continuous re-inoculation from the leakage. In this investigation both situations (persistent low cell density or sudden high cell density entry) are considered. However, increasing cell numbers in water could not be observed and only a slight increase was found in the sediment. *Ps. putida* is widely spread in the environment. With a number of closely related competitors and no selective pressure offering an advantage for the GEM, it seems plausible that increased growth would be difficult to achieve.

Nevertheless, persistence below the detection limit of 10 cfu ml^{-1} would not have been determined.

4.1.3.1 Viable But Non Culturable State (VBNC)

For the experiments described here, the so called viable nut non culturable (VBNC) state had a number of implications. A bacterial cell can be described as VBNC if it "fails to grow on the routine bacteriological media on which it would normally grow and develop into a colony, but which in fact is alive and metabolically active". If the GEM survives in a physically unstable condition, it may not be detected, neither by the cultivation approach used in this work, and perhaps not in the competitive PCR either. Oliver (2000) reports that 300 times the amount of DNA were necessary for a PCR signal detecting VBNC cells as compared with culturable bacteria. This was assumed to be due to the production of stress-induced proteins that either modified DNA supercoiling or blocked the DNA polymerase from binding to the DNA. It can be theorized that a GEM cell introduced into the microcosm enters VBNC state and is not detectable with PCR, either, although later, it may recover to be culturable and PCR-detectable again. The time period over which the microcosms were operated was longer than in most experiments of this kind. However, even longer experimental periods might be necessary to enhance the chance of recuperation from VBNC, if some cells were temporarily undetectable by PCR or cultivation.

4.1.4 GEM Impact on the Indigenous Microbial Community in Stream Microcosms

TGGE/DGGE have proven to be powerful tools to observe community structure based solely on the 16S rDNA sequences of the organisms in the sample (Muyzer *et al.* 1993, Muyzer & Smalla 1998), thus avoiding cultivation-derived artefacts. Separation of the 16S rDNA PCR products on a polyacrylamide gel plus the application of a thermogradient allow discrimination of sequences of the same length differing in only one base (Felske *et al.* 2001, Nübel *et al.* 1996, Rosenbaum & Riesner 1987). The resulting TGGE banding patterns are subject to bias owing to selectivity during DNA extraction procedures, due to potential preferential amplification during PCR, and chimera formation (Wintzingerode *et al.* 1997). Moreover, TGGE/DGGE can only show the predominant species present in the community. Of the estimated $<10^4$ genomes only bacterial populations that make up more than 1% or more of the total community can be detected by PCR-DGGE (Muyzer & Smalla 1998), this leaves a many phylotypes of the microbial community undetected. The profile may thus not reveal the "true" community composition and. However, TGGE/DGGE are well suited to

depict changes in the diversity of complex bacterial caused by disturbances (e.g. GEM influence, pollutants, temperature, grazing, pH). Eichner *et al*. (1999) have used TGGE analysis to determine structural changes in community patterns after a pollutant shock with and without the presence of a pollutant-degrading GEM. The effect of root exudates on a bacterial soil community contaminated with different levels of heavy metals has been investigated using TGGE/DGGE (Kozdrój & van Elsas 2000) as well as the impact of mercury and herbicides on the soil bacterial community (Rasmussen & Sørensen 2001, Engelen *et al*. 1998). Here, the effect of the presence/absence of the genetically engineered *Ps. putida* KT2442::mer73 on sediment and water communities was examined. Moreover, one microcosm was inoculated with the non-recombinant *Ps. putida* KT2440 to exclude an inherent effect of the inoculation of a *Pseudomonas putida* strain. The received 16S rDNA patterns were highly complex for water and even more so for the sediment samples, forming a firm foundation for interpretation. However, an effect of inoculation could not be detected.

4.1.5 Gene Transfer

4.1.5.1 Horizontal Transfer of Chromosomally Integrated Genes

Gene transfer usually requires the presence of mobile elements, e.g. a plasmid, bacteriophage or a transposon, that have the ability to take up DNA and release it in another cell in a form that it can be translated into protein. Conjugation, transduction and transposition have been studied and found to occur in the environment (Bogdanova *et al*. 2001, Davison 1999, Miller 2001). The uptake of naked DNA, both of chromosomal or plasmid origin, which is liberated if cells lyse has also been shown to be of environmental relevance (Lorenz & Wackernagel 1994). Chromosomal DNA, on the other hand, is generally regarded stable (Abebe *et al*. 1997), transfer is assumed to occur too rarely for detection (Sengeløv *et al*. 2001) and the bacterial genome viewed to be a safe place for genetic engineering. However, conserved chromosomal sequences such as DNA coding for ribosomal RNA could be relatively frequent targets for homologous recombination and thus serve as natural vehicles for horizontal gene transfer (Strätz *et al*. 1996, Arber 2000). This could e.g. affect gene transfer of the cassette in the new construct *Ps. putida* KT2440::mergfp47-32 which inserted into 23S rRNA. Insertions in most of the other constructs were found in double or multiple occurring genes, although the sequences of these genes were not as conserved as the 23S rRNA.

The microcosm experiments in this work provided the following possibilities for the dissemination of the chromosomally integrated genes: (I) The construction of *Ps. putida* KT2442::mer73 was accomplished by transposon mutagenesis. Although the transposase was

not transferred in the course, inner and outer 19 bp Tn5 ends were and might later be recognized by natural broad host range Tn5 transposase variants. (II) Inoculation into microcosms must have caused a great number of cells to lyse and thus huge amounts of free DNA were delivered into the microcosm available for transformation. Preliminary results of a quantitative PCR approach (for the method see Felske *et al*. 2001) with stream microcosm samples showed a discrepancy between bacterial density as determined by cultivation and chromosome equivalents by two orders of a magnitude (see section 3.1.8.3). Nielsen *et al*. (2000) found that although chromosomal DNA can persist in soil for weeks, if protected by soil minerals, it may not be transformable for the same length of time. However, in the experiments of this work cells were inoculated continuously and "fresh" DNA constantly renewed. Although *Ps. putida* has not been shown to enter a competence state, it can be transformed by chemical treatment or electroporation in the laboratory. Lorenz & Wackernagel (1994) summarize procedures by which DNA can be translocated into cells and they point out that environmental situations may be similar to those procedures. Osmotic shock experienced by the inoculants in our experiments when they were transferred to the microcosms could e.g. have rendered them receptive for the uptake of DNA. (III) The mercury resistance operon is widely spread among bacteria and some sequences highly conserved (e.g. *mer*A, Osborn *et al*. 1997, Felske *et al*. submitted). By homologous recombination the *mer* operon or parts of it could be exchanged if the GEM would take up a plasmid bearing the mercury resistance genes. However, this would remain a silent transfer that would not have been detected, unless the plasmid was transferred to the recipient. In this case, the mercury resistance genes coming from the donor or indigenous bacteria could not be discriminated. Furthermore, if the constitutive promoter regulating *mer*TPAB in the GEM was transferred by homologous recombination, a recipient with an inducible promoter could obtain constitutive expression. (IV) Bacteriophages have been shown to be abundant both in freshwater and marine environments (Miller 2001). Transduction has been a mechanism for maintaining an otherwise counter-selected phenotype in a continuous culture model (Replicon *et al*. 1995) and transduction frequencies in a freshwater environment have been shown to be enhanced in the presence of SPM, where bacteria and bacteriophages can aggregate (Ripp & Miller 1995). Free phage particles can be assumed to have been abundant also in the microcosms used in this study. These could have infected and lysed the donor, randomly producing *mer* operon-containing phage particles for which then there would have been a great possibility of transducing the recipient.

Although some of the above scenarios may appear highly speculative, none of them can be ruled out completely. Gene transfer mechanisms operating in the environment may be much more complex than anticipated on the basis of our present knowledge. Thus, the experimental proof of lack of gene transfer under the specified conditions cannot be replaced by theoretical considerations, however convincing they may be.

While the dissemination of genes from genetically engineered microorganisms (GEMs) is regarded as posing an unpredictable risk by some (Ashelford *et al*. 2001), for others it could represent a valuable means in bioremediation in providing a rather wide variety of microorganisms with the detoxifying or degrading capabilities in question (Barkay *et al*. 1993, de Lipthay *et al*. 2001). Gene transfer from *Ps. putida* KT2442::mer73 cannot be viewed as risk, since bacterial mercury resistance genes are ubiquitous and can be found even in non-contaminated environments (Osborn *et al*. 1997). The dissemination of the genes in mercury contaminated regions would aid biological mercury transformation and the site profit with regard to pollution severity.

4.1.5.2 Effect of Selective Pressure on Gene Transfer

Rensing *et al*. (2002) review the influence of selective pressure on the horizontal transfer of DNA in soil bacteria. The presence of mercury has for example been shown to increase the frequency of mercury resistance plasmids. Smit *et al*. (1998) could demonstrate greater occurrence of self-transmissible mercury-resistance plasmids in mercury amended soil and suggested that the gene-mobilizing capacity be enhanced as mercury stress was applied. Rasmussen & Sørensen (1998) also found higher occurrence of mercury resistance and of self-transmissible plasmids in mercury contaminated marine sediment compared to unpolluted sediment. A positive effect of selective pressure on the emergence of transconjugants could be shown for phenoxyacetic acid and the *tfd*A bearing plasmid pRO103 (coding for a 2,4-dichlorophenoxyacetic acid dioxygenase, de Lipthay *et al*. 2001).

These studies show that the application of a mercury selective pressure can increase the frequency of horizontal transfer of the mercury resistance genes, and was hence an adequate measure to promote transconjugants in the experiments of this work. Despite this fact gene transfer could not be observed in the experiments for this thesis.

4.1.5.3 Effect of Cell Density on Gene Transfer

That cell densities do play an important role in horizontal gene transfer has been recognized (see below). It is conceivable that for a gene transfer mechanism that requires cell to cell contact such as conjugation the chance for horizontal gene exchange would rise with

increasing cell densities. For filter mating fresh overnight cultures are commonly used and concentrated to increase the cell density on the filter. Accordingly, Ravatn *et al*. (1998) found decreasing transconjugant frequencies with lower cell densities. Fry and Day (1990) admonish against using low initial cell densities or too few donors because conjugal transfer frequencies were declining rapidly (in the epilithon) if densities were below 2×10^5 cfu cm^{-2}. They found the optimal donor to recipient ratio to be between 1:60 and 16:1. Donor densities used to study conjugal transfer to indigenous aquatic microorganisms or to bacteria within a pilot-scale percolating filter bed were ca. 10^5 or 10^6 cfu ml^{-1} (Ashelford *et al*. 2001, Barkay *et al*. 1993). For the experiments in this work this meant that gene transfer was expected in the more densely populated sediment rather than in the water column. If the continuous release of lower cell densities did not result in gene transfer, a cell shock with high cell density might possibly induce it. However, neither approach did result in the detection of transconjugants.

Transformation is dependent on cell-DNA contact frequencies which in turn are also increased with higher cell densities and DNA concentrations. However, other parameters may also play a crucial role in the transformation process, e.g. half life of free DNA in the environment in question and competence state of the recipient. These factors are reviewed by Lorenz & Wackernagel (1994). As addressed above, in this work a constant renewal of fresh DNA-molecules was probably provided from lysed inoculants of which some would have been transformable even if a great portion was degraded by DNase.

4.1.6 Mercury Resistant Community & Molecular Determination of Bacterial Density

The mercury resistant community in the microcosms was to be shown by a *mer*A specific PCR and subsequent separation of the amplicons with TGGE. PCR was also employed to determine bacterial densities in the microcosms using primers specific for the GEM *Ps. putida* KT2442::mer73. Both methods, however, did not yield the expected results in terms of PCR amplicon quality.

Both, *mer*A PCR and cPCR were tried here with samples from the second microcosm experiment, for which the sediment sample had been obtained from the Elbe River in October 2000. The sediment DNA looked sheared on agarose gel (data not shown), although the same extraction method was used as in the first microcosm experiment (July 2000) for isolation of good quality sediment DNA, and despite changing extraction parameters (such as the duration of bead beating), no distinct bands for genomic DNA could be obtained.

Both, the *mer*A PCR and the cPCR have been shown to work well with other (biofilm) samples (Felske *et al*. submitted, Felske *et al*. 2001), however, with decreasing DNA quality and especially in connection with possible sediment-derived impurities in the DNA sample, PCR sensitivity diminishes and gives way to non-specific products.

The *mer*A PCR-TGGE protocol used in this work was adopted from Felske *et al*. (submitted) who were able to monitor a mercury resistant bioreactor community with this approach. However, they had to optimise the PCR for the different biofilm participants as signals for all strains could only be obtained by using a rather complicated combination of PCR cycles and annealing temperatures. Although they could also identify new bands, the PCR was primarily aimed at detecting the known strains in the bioreactor. In the work presented here, however, the *mer*A PCR was performed with samples of an entirely different origin and thus probably with a completely different set of mercury resistant bacteria and their resistance genes (in the sediment mercury resistant bacteria were between 10^4 and 10^5 per g or 1-10% of the total bacteria, see Figure 3-10). The thermogradient acrylamide gels with water and sediment samples showed a high background in most of the lanes that could have been derived from unspecific PCR artefacts, however, if the diversity of *mer*A genes in the samples was grand and the number of each *mer*A type comparably low, the formation of distinct bands would have been more difficult. Hence, rather than being unspecific, the background may have been the result of many different *mer*A PCR products. Moreover, if the *mer*A genes found in the Elbe River samples would have been much different from the genes found in the bioreactor, the *mer*A PCR may have not been optimal for the Elbe River samples and PCR cycling may have needed improvement for these samples.

4.2 Ps. putida *constructs for Bioremediation of Mercury Contaminated Waste Water*

4.2.1 *Construct Ps. putida* KT2442::mer73

The mercury reducing GEM *Ps. putida* KT2440::mer73 was constructed in 1994 by Joanne Horn and co-workers and appeared a promising candidate for the use in bioremediation of mercury contaminated waste water on the basis of very high and constitutive mercury transformation in pure laboratory cultures. In this work, *Ps. putida* KT2442::mer73 was tested for its influence on indigenous bacterial communities in the microcosm experiments. In laboratory scale bioreactors, however, the strain did not establish well (von Canstein *et al*.

2002a), it was washed out quickly possibly owing to a relatively long lag phase during growth (also see Figure 3-29) and not to a general lack of biofilm forming capability, as the GEM did prove to form good biofilms in microdishes.

Localization of the transposed genes in this work revealed integration at the very 3' end of an alanyl-tRNA-synthetase, probably not disturbing the function of the enzyme and not interfering with the regulation of the next gene downstream, a transport protein of the major facilitator superfamily. Alanyl-tRNA-synthetase is an important enzyme in protein synthesis and only one copy of the genetic code for the enzyme can be found in the *Ps. putida* KT2440 genome. Moreover, aminoacyl-tRNA-synthetases are very specific (Gottschalk 1985) and no other aminoacyl-tRNA-synthetase could have taken over the function of the alanyl-tRNA-synthetase. Thus, it is highly unlikely that enzyme function would have been destroyed because the cell would not have survived.. Furthermore, by scrutinizing the insertion site it could be found that no frame shift occurred, however, stop codons upstream the *mer* operon would have terminated transcription of the alanyl-tRNA-synthetase. However, it cannot be ruled out completely that the gene insertion is in fact responsible for the deteriorated growth and thus for the lack of establishing an active biofilm in the bioreactors

4.2.2 Tn5 Transposition Target Site Selection

Tn5 belongs to the class I transposons, that perform non-replicative transposition using a cut-and-paste mechanism and creates 9 bp duplications in the target DNA. Tn5 derivatives have been described useful for random mutagenesis (Horn *et al*. 1994, Alexeyev *et al*. 1995). Nevertheless, although it has been shown that no specific DNA sequence is required for Tn5 transposition and that insertions occur at many different sites of the bacterial genome (e.g. Herrero *et al*. 1990, this work), class I transposons have also been observed to prefer target DNA with a certain similarity to the outer ends. The preferred sequence of Tn5 transposase for integration has been deduced to be in GNT $^{T}/_{C}$ $^{A}/_{T}$ $^{A}/_{G}$ ANC (G=guanine, T=thymine, A=adenine, C=cytosine, N=any of the four bases) from a total of 354 Tn5 insertions (Goryshin *et al*. 1998).

In this work, particular bases within the duplication could be found at higher frequencies (>60%) at base positions 1 (G), 4 (C), 6 (A), 9 (C), 11(G) downstream of the O-end. This observation was in full agreement with the preferred Tn5 target sequence except of the G at position 11, that was not part of the duplicated sequence. Other positions also harmonized with the preferred integration site: At position 3 thymine dominated with 47%, at position 5 an adenine or thymine could be found in 67% of the cases. Position 7 contained an A in 40%

of the sequences. The consensus sequence found by Goryshin *et al.* (1998) was clearly not an absolute requirement for transposition. They found many of their inserts at sequences different from the consensus sequence at one or more positions and this was the same with the constructs in this work. Still, the accumulation of higher base frequencies within the duplicated sequence was remarkable. However, among the 20 constructs that were analysed, the insertion sites were well distributed over the recipient genome.

4.2.3 Integration/Protein Functions Assigned to Ps. putida KT2440 Open Reading Frames

Although the *Ps. putida* KT2440 genome has now been fully sequenced (Nelson *et al*. 2002), accurate annotations for the DNA sequences are lagging behind. Often times assigned gene functions in the annotations have not been experimentally verified, rather similar DNA sequences have been found in databases for which a particular function could be elucidated (on the basis of experimental data or merely protein motifs). Misassignment during the annotation process owing to error propagation and a lack of experimental data cannot be ruled out (Peterson *et al*. 2001). Hence, annotated proteins whose functions have not been proven experimentally must be viewed with care. Furthermore, this demonstrates the need for experimental protein research that remains crucial if one wants to understand the meaning of the genetic data that has been obtained.

In general, transposon mutagenesis, selects for constructs that are viable. If transposition occurs within a gene that is essential but destroyed by the insertion, the strain will not be able to thrive and form colonies, thus lethal insertions are counter-selected. On the other hand the selection of the constructs with mercury required the mercury resistance to be expressed in adequate amounts. Transcription from a promoter that was sufficiently strong was thus compulsory for the strains to grow on the selection medium. Strong promoters, however, indicate a relative importance of the gene(s) they are regulating. It was thus not surprising that many of the insertions were found within genes, that are necessary for maintenance or house-keeping of the cell (see below: *aro*Q, *nuo*CD, *rrn*, *ilv*N & *ilv*B) and it raises a special interest in the identity of the hypothetical proteins. For a bacterium to grow despite disruption of a particular gene it either needs other genes with the same functions to surrogate the destroyed gene or the function of the disrupted gene was not (completely) destroyed by the insertion.

In this work, localization of the gene insertion in the constructs was carried out to partially characterize the newly created strains. Although many of the genetic modifications could indeed be elucidated, information about the kind of transcriptional control exerted on the

inserted genes was often lacking or incomplete, however, two of the insertions were found in RNAs (*Ps. putida* KT2440::mergfp47-30 & 47-32) that are usually transcribed from extraordinarily strong, growth-rate regulated promoters. High expression was also true for the GEM of Horn *et al*. (1994) that was used in this work for safety studies and for *Ps. putida* KT2440::mergfp46-7, 47-14 and 47-16. In these strains the integration occurred between the small and large subunits of the biosynthetic acetolactate synthase (*ilv*N and *ilv*B), within the coding region for an electron transferring subunit of the NADH dehydrogenase I (*nuo*CD) or within the 3-dehydroquinate dehydrogenase (*aro*Q) respectively for the three strains. The first and the latter are crucial in amino acid biosynthesis (isoleucine, leucine, valine/ phenylalanine, see 3.3.3 for discussion of *aro*Q insertion), while NADH dehydrogenase I is an essential component of the respiratory chain. The insertion into *nuo*CD in *Ps. putida* KT2440::mergfp47-14 occurred nearly 500 bp downstream of the 5' end of the 1800 bp gene. When growth of *Ps. putida* KT2440::*mergfp*47-14 in M9 medium with benzoate (data not shown) was compared with growth of the other constructs the strain showed indeed a prolonged lag phase which may be accredited to the insertion. However, this was a preliminary result which must be confirmed. Impeded growth of *Ps. putida* KT2440::*mergfp*47-14 could not be found on M9+benzoate agar plates. As the name indicates *nuo*CD comprises two functions. One can speculate that the insertion only impaired but did not fully destroy the function of the particular subunit or of the entire NADH:ubiquinone oxidoreductase.

Among the other construct strains growth in M9 (+benzoate) was quite similar and not deviant to the parent *Ps. putida* KT2440 (Figure 3-29). Besides the mercury reducing GEM *Ps. putida* KT2442::mer73 and the mercury reducing fluorescent *Ps. putida* KT2442::mer::gfp11, a delayed lag phase compared to the other constructs and the wildtype was also found in *Ps. putida* KT2442::mergfp46-2, 46-7 and 46-8, possibly a result of the insertion in these strains. Again, these are preliminary results, and effects during growth under different conditions (different medium, temperature, carbon source etc.) might reveal other effects of the gene insertion. As a link between genetic and physiological characterization, proteomics could be capable of yielding important information about the behaviour of the construct strains.

Differences among the strains could be found in the development of fluorescence during colony growth (see 3.3.5.1). Andersen *et al*. (1998) also observed a heterogeneous and changing distribution of the GFP fluorescence. Remarkably, they did not observe dark centres (as with *Ps. putida* KT2440::mergfp47-32 in this work), rather they found the centres to be

the brightest spots within colonies of *Ps. putida* KT2442 that had been trans-mutagenised with a GFP version (last three amino acids AAV, that had a similar half time to the 46 version (-ASV) used in this work. They speculated this to be caused by cannibalism of the cells in the centre keeping them metabolically active. In contrast they soon found brightest fluorescence at the borders of the colony in the strain carrying a GFP version with LAA at the last three positions (least stable GFP variant that was also used in this work).

Although the constructs in this work mostly showed bright centres, darker cores could sometimes be found after some time with the concurrent appearance of a ring pattern. The *gfp* cassette of Andersen *et al*. (1998) contained a LacI repressible promoter ($P_{A1/04/03}$) while the *mergfp* cassette used in this work was integrated into the host genome without transcriptional control of its own but was dependent upon host transcriptional machinery to be expressed. In *Ps. putida* KT2440::mergfp47-11 fluorescence was distributed homogenously during colony growth (Figure 3-32) and was present even in the old colony, while in *Ps. putida* KT2440::mergfp47-1, fluorescence concentrated in the centre of the colony and diminished after a few days. Sequence data about the genomic integration site could not reveal further clues as there was no good sequence for *Ps. putida* KT2440::mergfp47-1 and the annotation for *Ps. putida* KT2440::mergfp47-11 merely rendered a hypothetical protein without a hint about its function. As already described above many of the unambiguously identified genes were constitutively transcribed. However, the promoter is not known for all identified insertions. Differences in the transcriptional control could be made responsible for variations in fluorescence patterns within the colonies if cassettes were under the control of promoters active only during certain stages of the growth cycle. Metabolic activity is crucial for GFP expression especially with the destabilized GFP versions However, different transcriptional regulation may be responsible for differences in expression.

4.2.4 Fluorescence as a Reporter of Mercury Transformation

The visualisation of bacteria by virtue of GFP fluorescence has found wide application (Unge *et al*. 1998, Errampalli *et al.* 1999). Only some of the benefits are the non-invasiveness of the approach, the possibility for on-line or real time monitoring. Furthermore, GFP unlike Lux does not require a co-factor for fluorescence. Detection is relatively easy by fluorescence microscopy, fluorescence activated cell sorting (FACS) or spectrofluorometry.

Although useful for detection of bacterial cells, including those that are no longer metabolically active, stable GFP accumulates and may become a burden to the bacterial cell. Therefore, destabilized GFP variants were used in this work which possessed different

C-terminal tags conferring different susceptibilities to tail-specific proteases (Andersen *et al*. 1998). Three variants with different half lives were used to allow for a spectrum of fluorescence intensities of the transformant strains. Among the strains obtained in the modification one strain with a medium fluorescing GFP variant (46 series), and several strains with a relatively stable GFP variant (47 series) displayed fluorescence intensities that would render them easy for detection. In combination with a high mercury transformation rate, that in some cases was notably higher than that of *Ps. putida* KT2442::mer73, some of these strains are promising candidates to be used in bioremediation of waste water contaminated with ionic or organic mercury. Furthermore, fate, persistence and performance of a construct strain within a bioreactor biofilm could easily be monitored, without laborious molecular based methods.

Green Fluorescent Protein has been widely used as reporter gene for gene expression in mammalian (Kain *et al*. 1995) and plant cells (Blumenthal *et al*. 1999) as well as in prokaryotes (Southward & Surette 2002). In bacteria *gfp* has frequently been fused with a promoter of interest to study gene regulation but without the actual expression of the protein that is normally transcribed from this promoter. Targets of interest were e.g. promoter elements of heat shock stress proteins (transcription factor σ^{32}, protease subunit ClpB, DnaK; Cha et al. 1999) to study stress response in *E. coli* cells or p_{mer} and its regulatory gene *mer*R for the detection of bioavailable mercury using *E. coli* and *Ps. putida* (Hansen & Sørensen 2000). Furthermore, *gfp* has been fused with the growth-rate regulated *E. coli rrn*BP1 promoter to enable monitoring of the distribution of growth activities in flow-chamber biofilms (Sternberg *et al*. 2001). On the other side, simultaneous co-transcription of *gfp* and chloramphenicol acetyl-transferase (*cat*) has been achieved in *E. coli* to demonstrate the possibility of on-line monitoring of a fermentation product (DeLisa *et al*. 1999).

Here, the two genes *gfp* and *mer* were under control of the same host promoter in the construct strain, thus transcription of the two functions in each individual strain was linked and fluorescence intensity most probably reflected mercury transformation. However this should be experimentally confirmed for each individual strain during cell growth. In a preliminary experiment the correlation of mercury transformation and *gfp* fluorescence intensity was investigated. High mercury transformation rates were anticipated to be correlated with high fluorescence intensity. For the constructs with the shortest (41) and the medium half life (46) this correlation could be found, although differences in fluorescence and mercury transformation were only marginal for constructs from the 41 series. Among the constructs with the most stable GFP (47) the strain with medium mercury transformation rate

showed lowest fluorescence. The basis for this result needs to be investigated further. It is feasible to quantify the mRNA for GFP and mercuric reductase to gain more information on the transcription of the two. If transcription was unambiguously found to be equal, then the low correlation of fluorescence and mercury transformation activity must be due to differences on the translational level.

Differences between the *mer* operon and *gfp* on the transcriptional level are highly unlikely. Nevertheless, while fluorescence intensity is directly dependent on the number of GFP molecules in the cell, mercury transformation depends on a variety of factors including the energy balance of the cell (NADH can be a limiting factor in the reduction of ionic mercury). The cells were sampled in late logarithmic phase. However, growth is not fully synchronized, i.e. in late logarithmic phase some cells have already reached stationary phase while others are still in the physiological state of mid-logarithmic phase. It is possible that one culture contained more cells already in stationary phase than another culture. This would inevitably have lead to a reduction of mercury transformation. In stationary phase GFP degradation by the protease would also have been attenuated, hence more molecules would have remained in the bacterial cell, despite decreased biosynthesis of GFP. Mercury transformation rate and fluorescence intensity over a growth cycle will have to be monitored for the individual construct strains to elucidate the relationship between the two functions.

4.2.5 The Best Fluorescent, Mercury Reducing Constructs for Bioremediation in a Bioreactor

Clearly, the three strains of the 47 series (*Ps. putida* KT2440::mergfp47-1, 47-11, 47-32) were superior both in fluorescence and mercury transformation and exceeded the mercury transformation of the GEM *Ps. putida* KT2442::mer73. While *Ps. putida* KT2440::mergfp46-7 showed high fluorescence, mercury transformation rates were good but below those of *Ps. putida* KT2442::mer73. In the few physiological tests no defect of these strains could be detected when compared to the parent strain *Ps. putida* KT2440, except for *Ps. putida* KT2440::mergfp46-7 which seemed to have a longer lag phase when compared with the other strains.

Of these three strains, the function of the disrupted gene was undoubtedly revealed only for *Ps. putida* KT2440::mergfp47-32. Here, a rRNA gene was disrupted that, however, was present in the genome in multiple copies. The construct showed the highest fluorescence as well as the highest mercury transformation rate. On this basis, *Ps. putida* KT2440::mergfp47-32 would be the strain of choice to be used in a bioreactor. Since it has been shown that multi-

species biofilms are advantageous to mono-species biofilms (von Canstein *et al*. 2002a), it would be feasible to introduce the construct into a multi-species bioreactor in order to protect the community from abrupt mercury peaks which the construct should be able to cope with well due to its constitutive expression. The bioreactor community could be made of wildtype isolates/invaders or of other construct strains that in a concerted action would best be able to bioremediate mercury contaminated waste water.

The safety of the new construct strains was enhanced by using a fully sequenced safety strain as a parent, by choosing the variant without the rifampicin resistance of *Ps. putida* KT2442, by the full genetic elucidation of the insertions and through the provision of GFP as part of the insertion cassette for easier detection.

An important question towards the monitoring of the constructs in mercury reducing biofilms is the prospect of single cell detection. A number of Pseudomonads that occur in nature show (mostly greenish) autofluorescence (e.g. see fluorescence of the parent *Ps. putida* KT2440 in comparison to the GFP-strains, Figure 3-30 to Figure 3-32). Thus for GFP fluorescence to be useful for monitoring, it needs to be clearly discernable from the autofluorescence of other strains. Overnight liquid cultures (LB medium) of the constructs were observed under the microscope (×400, ×1000 magnification with the fluorescence microscope, data not shown), however, except for *Ps. putida* KT2440::mergfp47-32 who fluoresced brightly, fluorescence seemed rather weak for the other constructs of the 47 series, and of the 41 and 46 series only *Ps. putida* KT2440::mergfp46-7 could be detected with the fluorescence microscope. The non-GFP-fluorescent *Ps. putida* KT2440 or *Ps. putida* KT2442::mer73 could not be detected in this way. However, these were preliminary tests that need confirmation. In a bioreactor, the constructs would grow continuously and not in batch as the overnight cultures that were observed here. The possibility of single cell detection in a bioreactor biofilm needs further investigation of construct fluorescence during different growth phases and in continuous culture. Moreover, construct detection within a non-fluorescent community needs further investigation. However, using confocal laser scanning microscopy and an appropriate experimental design it might in principle be possible to monitor the mercury reducing activity of the new constructs in a non-destructive way in intact biofilm samples. In this way, information on gradients of activity and interactions between individual biofilm cells in multispecies biofilm might be obtained.

5 Summary

A **Stream Microcosm** was designed to carry out ecological experiments regarding the impact of *Ps. putida* KT2442::mer73, a Tn5 mutagenised, mercury reducing GEM, if introduced into native bacterial river water and sediment communities. Since discharge of a bioreactor containing the GEM would most likely enter a river, a stream microcosm was chosen as the model system. Streams are tremendously complex environments that cannot be modelled efficiently in a simple system. Therefore, a complex microcosm was designed for the experiments. Flow visualization revealed laminar to transitional flow suitable for these kinds of experiments.

Survival and Persistence of the GEM were monitored during long-term low density inoculation and shock inoculation by cultivation on selective agar plates. Long-term inoculation was achieved by connecting the microcosm with a chemostat. While there was a minimal trend of higher bacterial densities in the sediment, no long-term effect could be seen in the water. After a recycling period of 10 days, the microcosms were sampled for a total of 43 days. This is longer than most experiments of this kind were performed.

The **Bacterial Community** from water and sediment was profiled as 16S rDNA-PCR-TGGE banding pattern. An influence of the GEM on the bacterial community could not be detected.

Gene Transfer was monitored in a second microcosm experiment during which the microcosms were inoculated with the GEM and a tetracycline resistant *Ps. putida* KT2440 as close relative and thus probable recipient in a gene transfer event. This experiment was operated for 45 days after the recycling period including one microcosm that was continuously amended with PMA to create a selection pressure. However, gene transfer could not be detected.

The **Insertion Site** of the *mer* operon of *Ps. putida* KT2442::mer73 was elucidated by restriction digest and IPCR and subsequent sequencing of the neighbouring genomic DNA. This knowledge allowed the development of a quantitative, competitive PCR method for specific detection and quantification of the GEM.

New Mercury Reducing Fluorescent *Ps. putida* Strains were constructed for a possible use in bioremediation of mercury contaminated waste water by mini-Tn5 mutagenesis of a *mergfp* cassette into the genome of the safety strain *Ps. putida* KT2440. Three destabilized versions of GFP with different half lives were used in the construction. Of the obtained strains some were highly resistant to mercury exceeding the capabilities of *Ps. putida* KT2442::mer73. The integrated *mergfp* cassette should allow detection of the cell due to its green fluorescence, and monitoring of mercury reducing activity during bioremediation.

The **Insertion of the *mergfp* Cassette was Localized** in most of the new strains with the help of the information obtained from the *Ps. putida* KT2440 genome project. Most insertions had occurred within encoding regions for hypothetical or putative proteins. Of the disturbed codons that could be unambiguously identified, nearly all had multiple copies present in the *Ps. putida* KT2440 genome. An exception were *Ps. putida* KT2440::mergfp47-14 which carried the sequence within the gene for the NADH dehydrogenase which is crucial for respiration and *Ps. putida* KT2442::mer73 in which the insertion was within an alanyl-tRNA-synthetase. Despite the insertion the strains grew well, however, with a prolonged lag-phase.

The possibility to use **GFP Fluorescence as a Reporter for Mercury Transformation Activity** was investigated. A weak correlation between GFP fluorescence and mercury transformation could be found among the strains regardless of the promoter, however, this correlation would have to be proven for the individual strains in a growth experiment.
Ps. putida KT2440::mergfp47-32 was found to possess highest mercury transformation rates as well as highest fluorescence and was therefore the best strain among the constructs of this work. *Ps. putida* KT2440::mergfp47-1 and *Ps. putida* KT2440::mergfp47-11 also performed and fluoresced extremely well and are possible candidates for bioremediation in a mercury reducing bioreactor.

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Symbols and Abbreviations

AAS	atomic absorption spectroscopie
bp	basepairs
cfu	colony forming units
cPCR	competitive PCR
CTAB	hexa-decyl-trimethyl ammonium bromide
ed(s).	editor(s)
EDTA	ethylendiamine tetra acetate
e.r.a.	environmental risk assessment
et al.	*et alii/ et alia,* Latin for: and others
etc.	et cetera, Latin for : and so on
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
×g	times gravity
gfp	gene for GFP
GEM	genetically engineered microorganism
GFP	Green Fluorescent Protein
GMO	genetically modified organism
Hg	mercury
Hg^0	elemental mercury
Hg^{2+}	divalent ionic mercury
IPCR	inverse polymerase chain reaction
kb	kilobases
L	litre, length
LB	Luria Bertani Medium
mer	mercury resistance operon
mergfp	gene cassette consisting of *mer*TPAB and *gfp*
ν	kinematic viscosity
NADH	nicotinamide-adenine dinucleotide
NCBI	National Center for Biotechnology Information
OD_{600}	optical density at 600 nm
ORF	open reading frame
PCR	polymerase chain reaction

PMA	phenyl mercuric acetate
ppb	parts per billion [µg L^{-1}]
ppm	parts per million [mg L^{-1}]
Re	Reynolds number
Rif	rifampicin
SDS	sodium dodecyl sulfate
SSC	sodium chloride, sodium citrate
t	time
$t_{½}$	halflife
TE	Tris-EDTA
TIGR	Institute for Genomis Research
TGGE	thermo gradient gel electrophoresis
Tn5	transposon 5
U	velocity
v/v	volume per volume
wt	wildtype
w/v	weight per volume

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